Analysis and treatment of pathological blood-brain barrier dysfunction during experimental allergic encephalomyelitis

Paul, Carolyn

Award date:
1996

Awarding institution:
University of Bath

ANALYSIS AND TREATMENT OF PATHOLOGICAL BLOOD-BRAIN BARRIER DYSFUNCTION DURING EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

Submitted by Carolyn Paul
for the degree of PhD
of the University of Bath
1996

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Summary

Crucial to the development of multiple sclerosis and the animal counterpart experimental allergic encephalomyelitis (EAE) is loss of blood-brain barrier (BBB) integrity whereby systemic solutes and cells gain unregulated entry to neuronal tissue resulting in serious neurological sequelae. This thesis examines the control of EAE development through pharmacological restriction of BBB dysfunction with consideration of the mechanisms underlying the neurovascular perturbation.

A double radioisotope technique was developed to accurately and reproducibly quantitate BBB permeability to systemic albumin during the progression of EAE. Abnormal levels of protein transport were measured at the BBB concomitant with symptom onset in acute EAE. Disruption to the barrier was initially greatest in the spinal cord continuing throughout disease expression and persisting into early recovery where protein extravasation in the cerebellum was maximal.

Control of neurovascular permeability by dexamethasone (DEX), cyclosporin A (CSA) and FK506 was investigated following short-term dosing during both acute and recovery stages of EAE. DEX (0.1-1mg/kg), demonstrated a dose-dependent inhibition of neurovascular abnormalities which could be antagonised by administration of the anti-glucocorticoid RU38486. CSA (50mg/kg) partially suppressed abnormal neurovascular permeability in EAE following therapeutic administration and normalised barrier function when given during recovery. FK506 (10mg/kg) maintained BBB function within normal limits during both acute and recovery phases. Combined DEX and CSA therapy, using doses of minimal individual effectiveness, demonstrated improved suppression of neurovascular permeability.

Finally, administration of the N-methyl-D-aspartate receptor antagonist MK801 demonstrated a dose-related suppression of BBB permeability in acute EAE following both prophylactic and therapeutic dosing.

Effective doses and drug combinations for the control of barrier breakdown in EAE have been shown for DEX, CSA and FK506, which may be applicable to the treatment of other neurological conditions. Furthermore, a candidate pathway in which the N-methyl-D-aspartate receptor is pivotal has been implicated in the loss of neurovascular function during EAE.

Acknowledgements

I would like to thank my supervisor Dr Chris Bolton firstly for the opportunity to do this PhD and secondly for excellent supervision and valued friendship. I am grateful to the Multiple Sclerosis Society of Great Britain and Northern Ireland for financing the project.

I would also like to thank: all University of Bath Animal House staff past and present for their help, in particular Mrs Lesley Moore whose technical expertise were invaluable; Dr P. Christie and Dr M. Watson for statistical advice; Dave Hornsey for advice on the use and handling of radioisotopes; Dr G. Oliver for help with establishing a radioiodination procedure; Carolynn Male and staff in the Department of Cellular Pathology, Royal United Hospital, Bath for preparation of tissues for histology; Dr L. Notarianni for the use of facilities at the Wolfsen Centre, Royal United Hospital, Bath; group members Julie, Gwen and Pennie for helpful talks and permission to discuss aspects of their research; and the University's technicians and support staff for their hard work.

Many thanks to my parents, Tessa, Alison, Robert, the 'Cameron' clan, Jenan, Amber and Ulrike for their continued support and for 'believing'. Finally, a big hug and thank-you to my husband Andy for understanding 'the computer' and generating the graphics figures, but mostly for his unerring support.

Thank-you All.

Contents

Summary 2

Acknowledgements 4

Contents 5

List of Figures 10

List of Tables 13

Abbreviations 14

1. Introduction 17

1.1 The Mammalian Blood-brain Barrier 18

1.1.1 Location 18

1.1.2 Unique Structure and Characteristics 19

1.1.3 Normal Nutrient Transport 22

1.1.4 Immunological Access to the CNS 25

1.2 Abnormal BBB Function 27

1.2.1 BBB in Disease 27

1.2.2 Non-disease BBB Dysfunction 29

1.3 Multiple Sclerosis 29

1.3.1 Prevalence and Epidemiology 30

1.3.2 Clinical Presentation 31

1.3.3 Cellular Pathology 31

1.4 Experimental Allergic Encephalomyelitis 34

1.4.1 Model Development and Induction 34

1.4.2 Neurological Deficits and Pathology 36

1.5 BBB Breakdown During MS and EAE 37

1.5.1 BBB Pathology in MS 37

1.5.2 BBB Pathology in EAE 38

1.5.3 Ultrastructural Aberrations 40

1.5.4 Mechanisms of BBB Perturbation 41
1.6 Treatment of MS 42
1.6.1 Current Drug Treatments 43
1.6.2 Emerging Drug Treatments 45
1.6.3 Correction of BBB Dysfunction 46
1.6.4 Targeting the BBB in EAE 47
1.7 Aims of the Project 48
2. Materials and Methods 49
2.1 Animals 50
2.2 Inoculation for EAE 50
2.3 Clinical Assessment of EAE 51
2.4 Iodination of Rat Serum Albumin with ^{125}Iodine 52
2.5 Blood Volume Markers 53
2.5.1 ^{113m}Indium and ^{111}Indium Labelling of Transferrin 53
2.5.2 ^{111}Indium-tropolonate Labelling of Red Blood Cells 53
2.6 Quantitation of Blood-brain Barrier Permeability 54
2.6.1 Edema Measurement 54
2.6.1.1 Dual Label Determination of Water Extravasation 55
2.6.1.2 Ethanol Extraction of ^3H_2O 55
2.6.1.3 Tissue Weight 56
2.6.2 Albumin Extravasation 56
2.7 Drug Preparation and Administration 57
2.7.1 Dexamethasone 57
2.7.2 Cyclosporin A 58
2.7.3 FK506 58
2.7.4 MK801 59
2.7.5 RU38486 59

2.8 Histology 59
2.9 Corticosterone Radioimmunoassay 60
2.10 Creatinine Determination 61
2.11 Statistics 63
3. Development of an In Vivo Technique to Quantify BBB Permeability 65
3.1 Results 66
3.1.1 Protein Extravasation 66
3.1.1.1 ^{113m}In-Transferrin as a Blood Volume Marker 66
3.1.1.2 ^{111}In-Transferrin as a Blood Volume Marker 68
3.1.1.3 ^{111}In-Transferrin Circulation Times and Isotope Binding Efficiency 72
3.1.1.4 ^{111}In-RBC as a Blood Volume Marker 75
3.1.2 Water Extravasation 79
3.1.2.1 Dual-tracer Analysis of Edema Formation 80
3.1.2.2 Ethanol Extraction of $^{3}H_2O$ for Edema Measurement 80
3.1.2.3 % Water Content 88
3.2 Discussion 92
4. Characterisation of BBB Dysfunction during the Course of Acute EAE in the Lewis Rat 98
4.1 Results 99
4.1.1 Profile of Neurovascular Breakdown during Acute EAE 99
4.1.1.1 Body Weight Changes and Development of Neurological Deficits 99
4.1.1.2 BBB Dysfunction 99
4.1.2 Neurovascular Dysfunction: Comparison of Routes of Vehicle Administration 105

4.1.3 Neurovascular Dysfunction: Correlation with Neurological Signs 105
4.2 Discussion 109
5. Modulation of BBB Dysfunction by DEX Administration 113
5.1 Results 114
5.1.1 Therapeutic Administration of DEX in the Acute Phase of Disease 114
5.1.2 Administration of DEX during the Early Recovery Phase of Disease 118
5.1.3 Reversal of DEX Control by Administration of the Steroid Antagonist RU 38486 121
5.2 Discussion 124
6. Modulation of BBB Dysfunction by Immunosuppressive Agents Cyclosporin A and FK506 132
6.1 Results 133
6.1.1 Therapeutic Administration of CSA in the Acute Phase of Disease 133
6.1.2 Administration of CSA during the Early Recovery Phase of Disease 136
6.1.3 Therapeutic Administration of FK506 in the Acute Phase of Disease 139
6.1.4 Administration of FK506 during the Early Recovery Phase of Disease 143
6.2 Discussion 145
7. Combined Administration of Cyclosporin A and Dexamethasone during the Acute Phase of EAE 152
7.1 Results 153
7.2 Discussion 160

8. The Role of the N-methyl-D-aspartate Subtype of Glutamate Receptor in Neurovascular Dysfunction during Acute EAE 163
8.1 Results 164
8.1.1 Suppression of EAE by Administering the NMDA Receptor Antagonist, MK801, during the Effector Phase of Disease Induction 164
8.1.2 Suppression of EAE by Therapeutic Administration of the NMDA Receptor Antagonist, MK801 167
8.2 Discussion 172
Conclusions 179
Future Directions 181
References 183
Appendix - Radioisotope Physical Data 215
Publications 216

List of Figures

Figure1: Cross Section of the Blood-brain Barrier 20

Figure 2: Transport at the Blood-brain Barrier 23

Figure 3: Schematic Representation of MS Plaque Pathology 33

Figure 4: ^{125}I-Corticosterone Standard Curve 62

Figure 5: Standardisation of equipment for creatinine measurement. 64

Figure 6: Correction curve for ^{113m}In decay 67

Figure 7: Measurement of BBB permeability using ^{113m}In-transferrin to correct for blood volume. 69

Figure 8: Comparison of Normal ^{125}I BE values Measured in the Cerebellum. 70

Figure 9a-c: Measurement of BBB permeability using ^{111}In-transferrin to correct for blood volume. 71

Figure 10: Correction curve for ^{111}In decay. 73

Figure 11: ^{111}In-transferrin Circulation Times 74

Figure 12a-c: Measurement of BBB permeability using ^{111}In-RBC to correct for blood volume. 77

Figure 13: Final Activity of ^{111}In / 5 x 109 RBC Preparations 78

Figure 14: Standardisation of Dual Count Parameter for the Measurement of ^{3}H and ^{125}I Radioisotopes. 81

Figure 15: Water Extravasation During EAE Measured by a Dual-tracer Technique 82

Figure 16: Water Extravasation During EAE Measured by Ethanol Extraction Technique 85

Figure 17: Variability of Normal $^{3}H_2O$ Values in the Cerebellum following Ethanol Extraction. 86

Figure 18: Variability within Experiments when comparing Normal and EAE Values of Water Extravasation by the Ethanol Extraction Technique 87

Figure 19: Water Extravasation During EAE Assessed by % Water Content - Comparison of Normal and EAE at D7 PI. 89

Figure 20: Water Extravasation During EAE Assessed by % Water Content - Comparison of Normal and EAE at D12 PI. 91

Figure 21: Body Weight Profiles for EAE and CFA-inoculated Lewis Rats with Comparison to Normals. 100

Figure 22: Development of Neurological Symptoms in EAE-sensitised Lewis Rats. 101

Figure 23: Profile of Protein Extravasation in the CNS of CFA-inoculated Lewis Rats. 102

Figure 24: Profile of Protein Extravasation in the CNS during the Development of Acute EAE in the Lewis Rat. 104

Figure 25: Route of Vehicle Administration: Effect on the Permeability of the BBB to Protein 106

Figure 26: Correlation between Neurovascular dysfunction and Severity of Neurological Deficits in EAE-sensitised Lewis Rats. 108

Figure 27: Inhibition of BBB breakdown by DEX Administered During the Acute Phase of EAE 116

Figure 28: Comparison of Weight Profiles and % Weight Loss in DEX and Vehicle Treated animals. 119

Figure 29: The Effect of DEX on Abnormal BBB Function during Early Recovery in the Acute Model of EAE. 120

Figure 30: Effect of the Glucocorticoid Antagonist RU38486 on the Control of BBB permeability by DEX. 122

Figure 31: Inhibition of BBB breakdown by CSA Administered During the Acute Phase of EAE. 134

Figure 32: Analysis of Body Weight Changes and Plasma Creatinine Concentration in CSA Treated EAE-sensitised Animals. 137

Figure 33: The Effect of CSA on Abnormal BBB Function during Early Recovery in Acute EAE 138

Figure 34: Inhibition of BBB breakdown by FK506 Administered During the Acute Phase of EAE. 140

Figure 35: Analysis of Body Weight Changes in FK506 Treated EAE-sensitised Animals. 142

Figure 36: The Effect of FK506 (10mg/kg body weight) on Abnormal BBB Function during Early Recovery in Acute EAE. 144

Figure 37: Proposed Mechanism of CSA and FK506 Inhibition of IL-2 Stimulated T cell Proliferation 148

Figure 38: 0.1mg/kg DEX and 25mg/kg CSA, Combined Dose during the Acute Phase of EAE. 154

Figure 39: 0.1mg/kg DEX and 35mg/kg CSA, Combined Dose during the Acute Phase of EAE. 156

Figure 40: 0.1mg/kg DEX and 50mg/kg CSA, Combined Dose during the Acute Phase of EAE. 157

Figure 41: 0.01mg/kg DEX and 50mg/kg CSA Combined Dose during the Acute Phase of EAE. 159

Figure 42: Nitrite levels detected in CNS cytosol preparations by Greiss assay 165

Figure 43: Inhibition of BBB Dysfunction during EAE by Prophylactic Administration of MK801 from D7 PI 168

Figure 44: Comparison of Body Weight Profiles from Studies of Prophylactic and Therapeutic MK801 Administration during EAE. 169

Figure 45: Inhibition of BBB Dysfunction during EAE by Therapeutic Administration of MK801 from Weight Loss 170

Figure 46: NMDA Receptor 173

List of Tables

Table 1: Comparison of the Pathological and Immunological characteristics of EAE and MS 35

Table 2: ^{111}In-transferrin Correction of Blood Volume: Comparison between Normal and EAE-sensitised Lewis Rats 75

Table 3: ^{111}In-RBC Correction of Blood Volume - Comparison between Normal and EAE-sensitised Lewis Rats 79

Table 4: Assessment of CNS % Water Content in Normal and EAE-inoculated Lewis Rats at D7 and D12 PI 88

Table 5: Summary of Neurological Status, Histological Scores and Plasma Corticosterone Levels following DEX Administration in the Acute Phase of EAE 117

Table 6: Antagonism of DEX-mediated Suppression of EAE by RU38486: Summary of Neurological Status and Weight Loss 123

Table 7: Summary of Neurological Status, Histological Scores and Plasma Corticosterone Levels following CSA Administration in the Acute Phase of EAE 135

Table 8: Summary of Neurological Status, Histological Scores and Plasma Corticosterone Levels following FK506 Administration in the Acute Phase of EAE 141

Table 9: Summary of Neurological Deficits, Plasma Creatinine and Weight Loss following Joint Administration of CSA and DEX 155

Table 10: Polyamine levels detected in CNS blood vessel isolates 165

Table 11: Summary of Neurological Signs, Percentage Change in Body Weight and Plasma Corticosterone Levels in EAE-sensitised Lewis rats following MK801 Administration 166

Abbreviations

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BE	Blood equivalents
C	Cerebellum
CFA	Complete Freund's adjuvant
CHLP	Complete hindlimb paralysis
CNS	Central nervous system
cpm	Counts per minute
CSA	Cyclosporin A
CSF	Cerebrospinal fluid
CSp	Cervical spinal cord
DEX	Dexamethasone
dpm	Disintegrations per minute
D 'x' PI	Day 'number' post-inoculation
EAE	Experimental allergic encephalomyelitis
EVBE	Extravascular blood equivalents
FT	Flaccid tail
Gd-DTPA	Gadolinium-dietheylene-triamine pentaacetic acid
$^{3}H_2O$	Tritiated water
HCl	Hydrochloric acid
HLPP	Hindlimb partial paralysis
HLW	Hindlimb weakness
^{125}I	^{125}Iodine
ICAM	Intercellular adhesion molecule

IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
^{111}In	^{111}Indium
^{113m}In	^{113m}Indium
i.p.	Intraperitonealy
i.v.	Intravenously
L-DOPA	L-dihydroxyphenylalanine
LSp	Lumbar spinal cord
MBP	Myelin basic protein
MNS	Mean Neurological Score
MP	Medulla-pons
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NAA	Neutral amino acid
Na^+-K^+	Sodium-Potassium
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NSD	No significant difference
ODC	Ornithine decarboxylase
PA	Polyamine
PBS	Phosphate buffered saline
PE	Plasma equivalent
PI	Post-inoculation
RBC	Red blood cells
RIA	Radioimmunoassay
RSA	Rat serum albumin
s.c.	Subcutaneously

SD	Standard deviation
SQP(E)	Spectral quench parameter (external)
TNF	Tumour necrosis factor
TSp	Thoracic spinal cord

1.
Introduction

1.1 The Mammalian Blood-brain Barrier

Central to the successful control of co-ordination and function of the higher vertebrate is the maintenance of a homeostatic cerebral environment in which neurological cells can perform their critical processing. In order to achieve a stable balance the central nervous tissue must remain distinct from the circulating blood plasma which is subject to both dietary and metabolically-mediated fluctuations in solute and ion concentrations.

1.1.1 Location

Studies recognising the unique isolation of the brain from the circulatory system have been regularly reported since the initial findings of Paul Ehrlich in the late nineteenth century. Ehrlich, who administered vital dye intravenously (i.v.) into laboratory animals noting that all internal organs but the brain took up the stain, concluded that the cerebral tissues lacked the cellular constituents necessary to absorb the dye. However, in 1913 Goldmann demonstrated that injection of dyes directly into the central nervous system (CNS) resulted in the colouration of neurological tissue alone with no dye entering the circulation to stain other organs. Therefore, the existence of a barrier between the brain and the bloodstream was inferred. The actual cellular location of the blood-brain barrier (BBB) remained a controversial topic until Reese, Karnovsky and later Brightman, performed a series of electron microscopy experiments during the 1960's employing the electron dense tracers horseradish peroxidase and microperoxidase (Reese & Karnovsky, 1967; Brightman & Reese, 1969). The tracers showed that transport between the blood and the brain was halted at the capillary endothelial cell tight junctions, with no inter- or intra-cellular movement apparent, leading to the conclusion that the anatomical site of the BBB is the cerebrovascular endothelial cell.

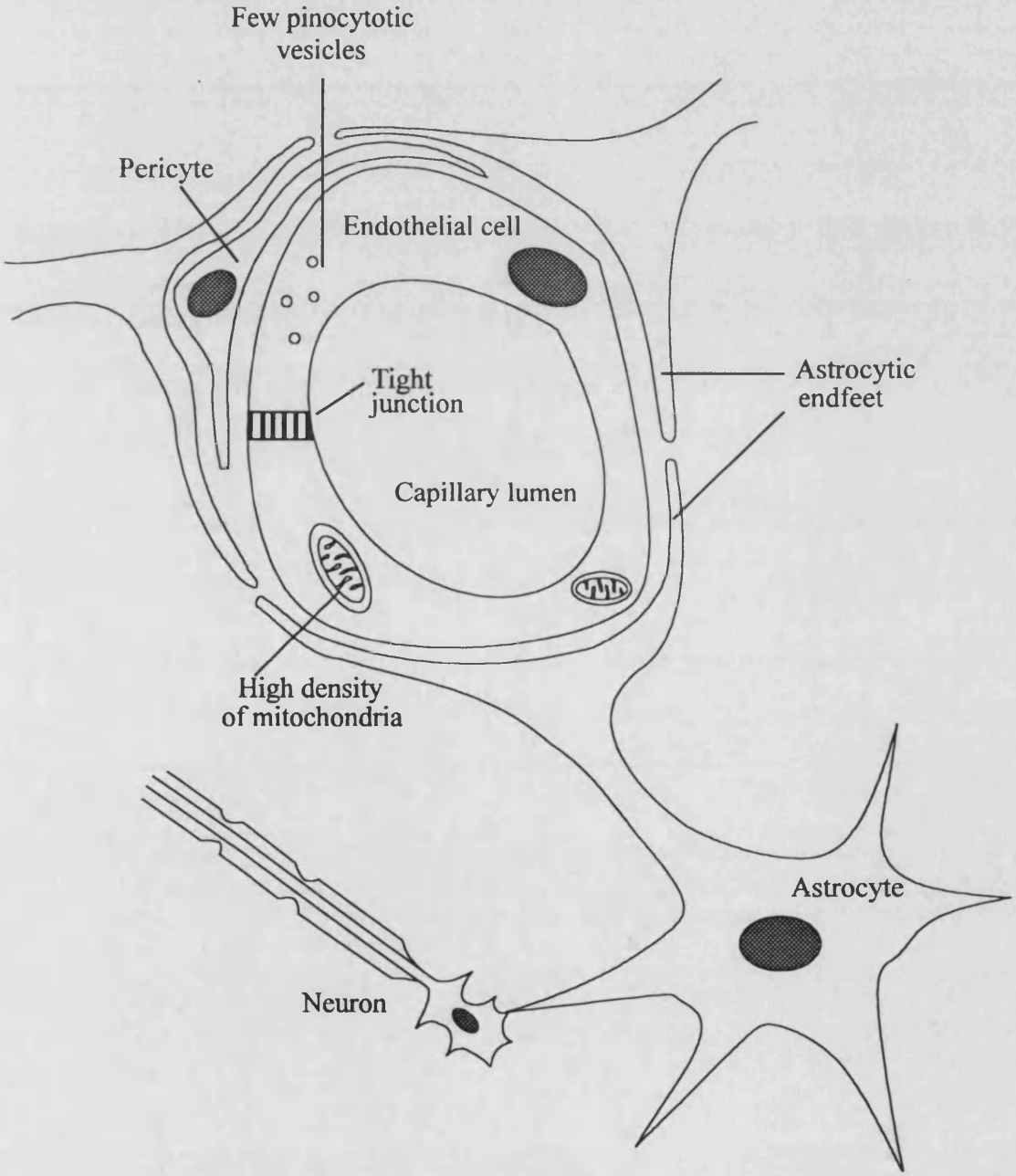
Collectively, within the CNS there are four types of barrier, not all located at the endothelium. They comprise: the endothelial BBB of the microvasculature (represented in Figure 1); the tight epithelia of the choroid plexus; the complex cellular arrangements of the meningeal compartments; and the ependymal tanycyte cells of circumventricular organs. Of the latter three (reviewed by: Krisch *et. al.*, 1978, 1984; Van Deurs, 1980) the choroid plexus is of particular importance as the epithelial structure represents the blood to cerebrospinal fluid (CSF) barrier, responsible for the active secretion of 66% of the total CSF. By comparison, secretion at the endothelial BBB into the cerebral parenchyma occurs at a much lower rate. However, with a surface area 5000 times that of the choroid plexus and with individual neurons being no further from a capillary than 40-50μm the BBB directly serves 99% of the cerebral tissue (Scharrer, 1944; Crone, 1971; Brightman, 1977). Therefore, within the CNS the capillary endothelium is the main barrier structure exerting control over the local neural environment.

1.1.2 Unique Structure and Characteristics

Distinct from the general characteristics exhibited by the vascular endothelium elsewhere in the body (Renkin, 1977), the endothelial cells of the neuro-microvasculature possess unique properties which enable highly selective control to be exerted over solute entry into the CNS interstitial fluid. These specialised endothelial cells do not develop from the neuroectoderm, but evolve from invading capillary cells originating outside of the CNS, differentiating in response to the neural environment. The transplant experiments of Stewart & Wiley (1981) clearly showed that grafted neural tissue could induce angiogenesis and BBB characteristics in host-derived non-cerebral endothelial cells. The main source of the BBB-defining factors has since been recognised as the type I astrocyte (Janzer & Raff, 1987). The interdigitating astrocytic sheath, unique to the microvasculature (Figure 1), is implicated in embryonic barrier induction as its formation accompanies tightening of

Figure 1: Cross section of the blood - brain barrier

The schematic diagram illustrates the complex and unique cellular arrangement associated with the BBB. Capillary endothelial cells are joined by a continuous belt of non-fenestrated tight junctions to occlude intercellular transport. Intracellular movement of solutes is highly regulated as indicated by the large number of mitochondria and limited pinocytotic vessicles viewed ultrastructurally. Astrocytic endfeet surround the endothelial cells, influencing BBB characteristics and creating a cellular link between the endothelium and the neurons.



the BBB during development (Senjo *et. al.*, 1986). Indeed, *in vitro* culture of cerebral capillary endothelial cells is significantly improved by co-culture with astrocytes or astrocyte-conditioned media, which increase the expression of BBB characteristics (DeBault & Cancilla, 1980; Tao-Cheng & Brightman, 1988).

By the first trimester of foetal life the endothelia form a continuous belt of occluding tight junctions which have 2-6 fusion points between cells and which lack fenestrations. These junctions preclude intercellular movement through the barrier as demonstrated by studies employing hydrophilic tracers horseradish peroxidase, cytochrome C and microperoxidase of molecular weights 40,000, 17,000 and 1,800Da respectively (Reese & Karnovsky, 1967; Brightman & Reese, 1969; Reese *et. al.*, 1971; Milhorat *et. al.*, 1973). Pinocytotic activity is evident in the endothelia of the majority of organs. However, Reese & Karnovsky (1967) and Clawson *et. al.* (1966), using peroxidase and colloidal iron respectively, demonstrated an absence of vesicular uptake activity in the brain under normal conditions. A paucity of pinocytotic vesicle transport combined with the lack of junctional fenestrae places a great restriction on the bulk flow of solutes across the barrier. In addition the high electrical resistance exhibited by the tight cerebro-endothelium ($2000\Omega.cm^2$, compared to $430\Omega.cm^2$ in the perineurium in the frog) further restrains blood-born solute entry into the CNS extracellular fluid (Crone & Olesen, 1982).

Histochemical analysis has recognised a number of identifying enzymes present in high concentration in the cerebral endothelium. These include Na^+-K^+-ATPase, γ-glutamyl transpeptidase, butyrylcholinesterase, alkaline phosphatase and aromatic-L- amino acid decarboxylase (Betz *et. al.*, 1980; Kreutzberg & Toth, 1983; Joó, 1985). Many of the enzymes are connected with the regulation of transendothelial movement and in conjunction with the abundant mitochondrial presence represent an active metabolic barrier within the physical BBB (Oldendorf & Brown, 1975).

1.1.3 Normal Nutrient Transport

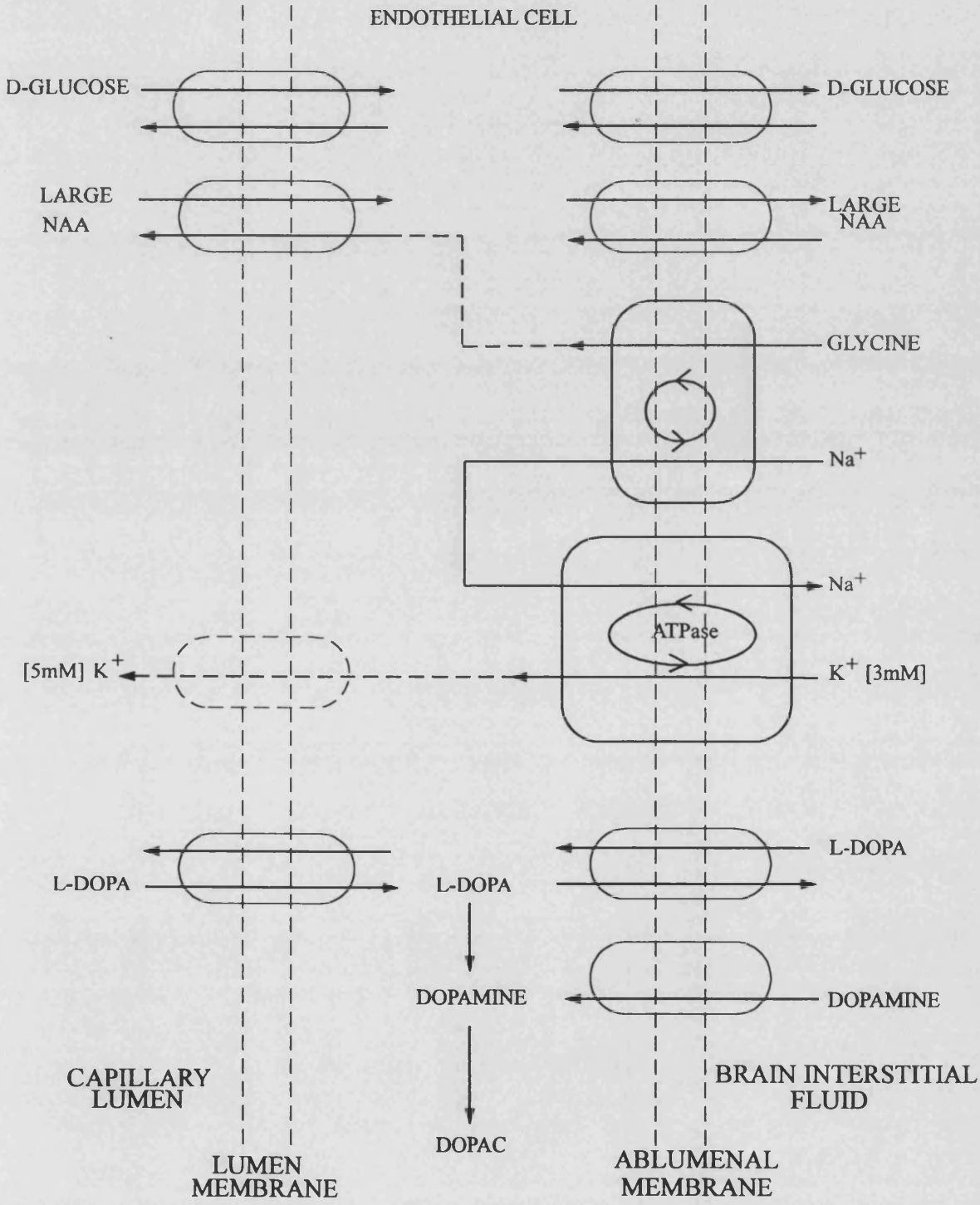
So far, the picture is of an impenetrable barrier, functional from birth, protecting the neural environment from contact with circulating plasma constituents. It is particularly important to shield the CNS tissues from the fluctuating levels of solutes and ions which the blood experiences during periods of active digestion, exercise and illness. Moreover, the circulation carries with it hormone and amino acid neurotransmitters whose unregulated entry into the CNS would be highly detrimental. Oldendorf (1971) has demonstrated that under normal physiological conditions the uptake of many of these compounds into the cerebral environment is low if not negligible. The only group of molecules to readily gain access to the neural parenchyma are lipid soluble compounds including alcohol, nicotine and heroine which explains the rapid action of these chemicals. It is however necessary for a number of non-lipid soluble molecules to reach the extracellular compartment of the brain and a range of selective transport systems exist to satisfy the metabolic and protein synthesis demands in addition to the homeostatic requirements of the central nervous tissue (Figure 2) (Bradbury, 1984).

The CNS requires glucose concentration to be maintained at 60-80% of the plasma concentration, from which cerebral oxidative metabolism can be sustained. The influx and efflux of the hexose is assisted by a self-saturable facilitated diffusion system that demonstrates a high degree of stereospecificity extracting only D-glucose from the blood (Crone, 1965; Betz *et. al.*, 1978). The non-metabolizable D-glucose analogue, 3-O-methyl-D-glucose, readily penetrates the BBB using the glucose carrier-mediated system, indicating that metabolism by the neural tissues is not a prerequisite for transport (Oldendorf, 1971).

Amino acid transport is dependent on the chemical side groups present and of particular interest are the large neutral amino acids (NAA) and the small NAA (Oldendorf & Szabo, 1976; Betz & Goldstein, 1978). The CSF protein concentration is normally only 0.01% that of the plasma because systemic proteins do not usually

Figure 2: Transport at the Blood - brain Barrier

Movement of nutrients, ions, and waste products across the BBB is highly organised. The D-glucose and large neutral amino acid (NAA) transporters facilitate normal osmotic diffusion. Potassium and glycine are actively moved unidirectionaly from the CNS to the blood. L-DOPA entering the endothelium from the circulation is metabolically converted to forms unable to cross the albuminal membrane. Broken arrow or transporter lines indicate hypothetical routes. (Adapted from a review of the BBB by Goldstein & Betz, 1986)



(Where : DOPAC = Dihydroxyphenylacetate)

traverse the BBB or the blood-CSF barrier. Large NAA are therefore required to cross the neurovasculature to satisfy the requirements of protein and neurotransmitter synthesis. The large NAA compete for a specific carrier-mediated transporter that is located in both the luminal and abluminal membranes of the endothelial cell so providing bi-directional transport as for glucose. Small NAA are not transported from blood to brain as the CNS has the capacity to synthesise them. However, the concentration within the brain interstitial fluid of selected members of this group, such as the potent inhibitory neurotransmitter glycine, require strict maintainance. Therefore, the cerebroendothelium has a small NAA carrier system located in the abluminal membrane to provide active unidirectional transport out of the CNS. Further carrier mediated systems exist at the BBB for monocarboxylic acids, nucleosides, purines and amines, while the circulating peptides insulin and transferrin rely on receptor-mediated endocytosis (Fishman *et. al.*, 1987; Broadwell, 1989; Pardridge, 1991).

Cerebral levels of the potassium ion must be maintained at 3mM in comparison to a vascular average of 5mM to prevent the unregulated firing of nerves by exceeding threshold potentials. Neurovascular control is exerted through a saturable active process driven by a Na^+-K^+-ATPase pump asymmetrically located in the abluminal membrane (Jones & Keep, 1987). Potassium is transported from the brain into the endothelium as sodium is simultaneously transported out into the extravascular spaces from where it is recycled by coupling to the glycine transport system. In spatial conjunction with the ion pump is a membrane specialisation found at the glio-vascular interface of the abutting astrocytic endfeet termed the 'orthogonal arrays of particles' (Anders & Brightman, 1979). The 'orthogonal arrays of particles', of undefined function, are distributed in a similar pattern to that of the potassium ion conductance over the glial cell surface. Also, their formation corresponds with the timing of increased homeostatic regulation of potassium at the BBB observed during embryo development, supporting the hypothesis that they correspond to symmetrical

high conductance potassium channels (Anders & Brightman, 1979; Newman, 1985; Jones & Keep, 1987).

In addition to regulation by selective transport, the BBB can exert metabolic control over the entry of certain substances into the CNS. The neurotransmitters dopamine and norepinephrine have a low brain uptake index (Oldendorf, 1971), however the precursor of these molecules dihydroxyphenylalanine (L-DOPA) can readily enter the cerebroendothelium through the large NAA carrier. Once internalised L-DOPA is enzymically modified firstly to dopamine by L-aromatic amino acid decarboxylase and then by monoamine oxidase to dihydroxyphenylacetate, neither of which can cross the abluminal membrane. Dopamine does have a specific carrier system located in the antiluminal plasmalemma, but the transport is unidirectional directing movement out of the brain parenchyma only (Betz & Goldstein, 1986).

Some provision is made within the cerebral capillary walls to control the otherwise unrestricted entry of some lipophilic compounds. The transmembrane glycoprotein, P-glycoprotein is the product of the multidrug resistance gene whose expression has been demonstrated in BBB capillary endothelial cells (Cordon-Cardo *et. al.*, 1989).The function of P-glycoprotein would appear to involve the restriction of transport of highly lipophilic toxic substances across the neurovasculature by an energy dependent efflux pump (Hamada & Tsuruo, 1988). Drugs, such as anthracyclins and epipodophyllotoxins that are used in tumor treatment, are particularly prone to the restrictive actions of this protein (Fojo *et. al.*, 1987).

1.1.4 Immunological Access to the CNS

The CNS has traditionally been viewed as an immunopriveledged site based on the extended survival time of skin allografts on the brain surface (Medawar, 1948). In support of this theory, the CNS lacks a true lymphatic system, possesses few immunocompetent cells, demonstrates only a limited expression of class II (Ia) major

histocompatibility molecules and contains soluble immunoregulatory factors. However, isolation of the CNS from the immune system is not complete.

While the BBB presents a physical barrier to humoral immune factors and cell entry it has become apparent that the CNS is routinely surveyed by T cells. Access to the CNS compartment requires T lymphocytes to be in an active state, although the antigen-specificity displayed does not appear to influence extravasation. Lymphocytes have been demonstrated in low but consistent numbers in the connective tissue of the meninges and within the parenchyma of normal human and rodent brain tissues (Booss *et. al.*, 1983; Lassmann *et. al.*, 1986). The neurovascular endothelial cells are capable of presenting lymphocyte adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), on the luminal cell membrane (Fabry *et. al.*, 1992). Whether ICAM-1 expression is involved in routine cell surveillance is uncertain, but it is clear that adhesion molecules can be upregulated in response to proinflammatory cytokines. Other immunological cells such as macrophages appear absent from normal adult rat brain (Sminia *et. al.*, 1987), which may explain the presence of the resident phagocytic microglia. Withdrawal of the surveillance cells from the CNS is believed to occur via the subarachnoid space, which serves some nerve routes and drains into the peripheral lymph system (Wekerle *et. al.*, 1986; Cserr & Knopf, 1992).

Major histocompatibility molecule class I expression is ubiquitous within the nervous system, but the expression of Ia is controversial. The endothelial cells of the BBB are beleived capable of Ia^+ presentation, but reports have varied and positive results have been attributed to the close perivascular proximity of Ia^+ locally resident monocytes (Sobel *et. al.*, 1987; Fabry *et. al.*, 1990; Lassmann *et. al.*, 1986). Strong evidence of Ia^+ cerebroendothelia was published by Wilcox *et. al.* (1989) who demonstrated constitutive Ia expression at the BBB of normal guinea-pigs with additional major histocompatibility epitope recruitment during CNS inflammation. The cerebroendothelial barrier is therefore intimately involved in immune cell recruitment. Interactions between the CNS and the immune system are frequent but

the immune reponse is modified thereby limiting the damage caused to the vulnerable neural tissue which has only a low regenerative capacity.

The BBB has been shown to play a crucial role in the maintenance of neural homeostasis and possesses a range of unique physical properties, transport systems and enzymic controls facilitating this function. The cerebral environment is reliant on the continuing strict regulation of the barrrier and can be markedly affected if the neurovasculature dysfunctions.

1.2 Abnormal BBB Function

1.2.1 BBB in Disease

Any medical condition which induces a breakdown of normal BBB function, whether related to physiological imbalance or disease, can lead to a range of highly detrimental neurological deficits. Symptoms may include impaired vision, muscle tremor, decreased mobility, headache, muscle stiffness and confusion. Examination of the CSF of a patient exhibiting signs of neurological impairment will generally show an elevated total protein concentration and in many infections a significant leukocyte count.

A pathological increase in CSF protein content can indicate:

1. dysfunction at the level of the BBB;
2. an increase in the plasma concentration of a specific protein such as transferrin;
3. a decreased CSF flow due to a spinal obstruction or blockage of the arachnoidal villi which allow CSF drainage into the venous blood supply;
4. an increase in the local synthesis of proteins within the CSF by either brain cells or invading inflammatory cells, such as local immunoglobulin (IgA) production during *Tuberculosis meningitis* (Reiber, 1986).

A general dysfunction in the regulatory capabilities of the BBB can be demonstrated by i.v. administration of radiolabelled albumin (Cutler *et. al.*, 1967; Leibowitz & Kennedy, 1972). Albumin acts as a good permeability marker as it is synthesised exclusively outside the CNS. Therefore, any labelled compound detected within the brain extravascular space or CSF would indicate an abnormal barrier function (Cutler *et. al.*, 1967). Unrestricted BBB dysfunction may be caused by increased pinocytotic activity, separation of endothelial tight junctional complexes and inflammatory cell entry into the CNS, which were shown to occur during the course of experimental bacterial meningitis (Quagliarello *et. al.*, 1986). The resultant bulk flow of plasma constituents across the capillary wall leads to the extravascular accumulation of vasogenic edema common to many neurological conditions including metastatic brain tumors, purulent meningitis and head injury (Beggs & Waggener, 1976; Grieg, 1988; Tunkel & Scheld, 1993). In addition, entry of primed leukocytes may result either in the formation of cellular perivascular cuffs and lesions characteristic of multiple sclerosis (MS) or in the purulent CSF typical of bacterial meningitis (Adams, 1976; Tunkel & Scheld, 1993).

BBB dysfunction need not cause an imbalance in the extracellular concentration of all plasma constituents but may be limited to specific transporter irregularities. In Alzheimer's disease a decrease in the cerebral metabolic rate for glucose occurs coincidentally with a marked reduction in the glucose transporter density (Harik, 1992). Lowered CSF glucose concentrations are also noted for meningitis, haemorrhage, cancer and spreading depression (Feldman, 1989). Other pathological cerebroendothelial cell aberrations include altered NAA transport during hepatic encephalopathy (James & Fischer, 1981), irregular expression of alkaline phosphatase on the abluminal as well as the luminal endothelial membrane during head injury (Pardridge, 1991) and stroke-related dysfunction of the choline carrier-mediated transport system (Kang *et. al.*, 1990).

1.2.2 Non-disease BBB Dysfunction

Experimental models of abnormal BBB function include focal cold injury, hyperosmotic barrier opening, heat stress, head trauma and acute hypertension (Trout *et. al.*, 1986; Koenig *et. al.*, 1989a; Sharma *et. al.*, 1992; Shuka *et. al.*, 1993; Shapira *et. al.*, 1993). While the stimuli are different, studies indicate that there are a number of shared features which may prove indicative of BBB dysfunction generally. On a broad scale increased water influx and plasma protein extravasation are typical of all models, although not all demonstrate increased pinocytotic activity (Petito & Levy, 1980; Trout *et. al.*, 1986). Biochemically, abnormal production of polyamines (PA) by ornithine decarboxylase (ODC) within the endothelial cells in both osmotically and cryogenically injured brain has been causally linked to barrier dysfunction (Koenig *et. al.*, 1989a,b). Furthermore, during cold-induced barrier perturbation the activity of Na^+-K^+-ATPase is impaired, as shown by reduced enzyme affinities for Na^+ and K^+ (Averet *et. al.*, 1984). As Na^+-K^+-ATPase is important in the maintenance of cell osmotic pressure, diminished function suggests a causal role in intracellular water uptake.

Neurovascular function during disease and under experimental conditions can be severely impaired. While the initial stimuli and resultant pathologies may vary, the observations available indicate that many conditions could have dysfunctional mechanisms in common, which would provide important sites for potential pharmacological correction.

1.3 Multiple Sclerosis

A neurological disease in which loss of BBB integrity is of paramount importance is MS (Grossman *et. al.*, 1988; Koopmans *et. al.*, 1989; Poser, 1992). While uncertainty stills exists regarding the epidemiology and mechanism of disease

onset, it is clear that an improved understanding of the pivotal stages of pathogenesis which culminate in expression of neurological deficits may improve therapeutic options.

1.3.1 Prevalence and Epidemiology

MS is typically an unpredictable chronic disease characterised by acute exacerbations and spontaneous remissions although acute, progressive, benign and subclinical forms may also occur (McDonald & Halliday, 1977). The nature of the condition is still contested between infection, autoimmunity or a combination of the two. However, neither the presence of an infective agent nor the conclusive identification of a trigger antigen have been elucidated to date (McFarlin & McFarland, 1982a; Rodriguez, 1989). MS is predominantly a neurological condition of young adults reaching a peak onset during the thirties and with a bias towards the female population. 'High risk' areas for MS prevalence include Britain and the rest of northern Europe with 100/100 000 individuals affected, while areas of 'low risk' include Africa and Asia, where 5/100 000 present with the condition. The 'risk' factor may be linked to the environment or a common agent within the environment as migration studies have indicated that peoples moving between high and low risk areas carry with them the exposure risk gained before the age of 15, which also implies that puberty plays an important part in disease acquirement (Acheson, 1977). Exposure to a viral infection is popularly believed to represent the 'risk' although what virus or group of viral agents are involved and whether their involvement is destructive or protective has not been proven. MS prevalence appears to be high in Caucasian groups and those populations which have been historically associated with European invasion or colonisation (Poser, 1992). In conjunction with information showing MS to be rare in black Africans and the Japanese, a genetically derived susceptibility to the condition would appear to be indicated. Indeed, familial studies have indicated an increased risk for close family members particularly for twins, although being a

monozygotic sibling of an MS patient is not conclusive proof of the disease occurring (Ebers & Bulman, 1986). There is now good evidence that the histocompatibility antigens HLA-A3, B7, DR2 and DW2 occur at a significantly higher frequency in MS patients of European white decent than in controls. However this is not true of other ethnic groups suggesting that the HLA system is not alone in controlling the genetic transmission of MS susceptibility (Batchelor *et. al.*, 1978).

1.3.2 Clinical Presentation

Clinically MS is highly variable, not only in its course of progression but also in the symptoms presented which vary from optical neuritis and ataxic gait to speech defects and spastic weakness. A patient's clinical status is defined by measurement of disability in line with the Kurtzke disability state scale, or other recognised grading system (Kurtzke, 1983). Diagnosis is difficult in the early stages as initial symptoms may be unremarkable and easily forgotten once remission occurs. Clinically definite disease is established after a minimum of two episodes that display differing symptoms which indicate the occurrence of lesions at two or more clinically separate sites within the CNS and with no other explanation of the observed abnormalities (McDonald & Halliday, 1977). Observation of neurological deficit is backed up by CSF analysis which generally demonstrates a mild increase in protein and lymphocyte levels plus a raised immunoglobulin concentration (Tourtellotte *et. al.*, 1984; McLean *et. al.*, 1993). Also magnetic resonance imaging (MRI) may now be used as a confirmatory tool to scan the CNS highlighting areas of abnormal composition which relate to lesion development (Papadopoulos *et. al.*, 1987; Grossman *et. al.*, 1988; Koopmans *et. al.*, 1989; Müller *et. al.*, 1989).

1.3.3 Cellular Pathology

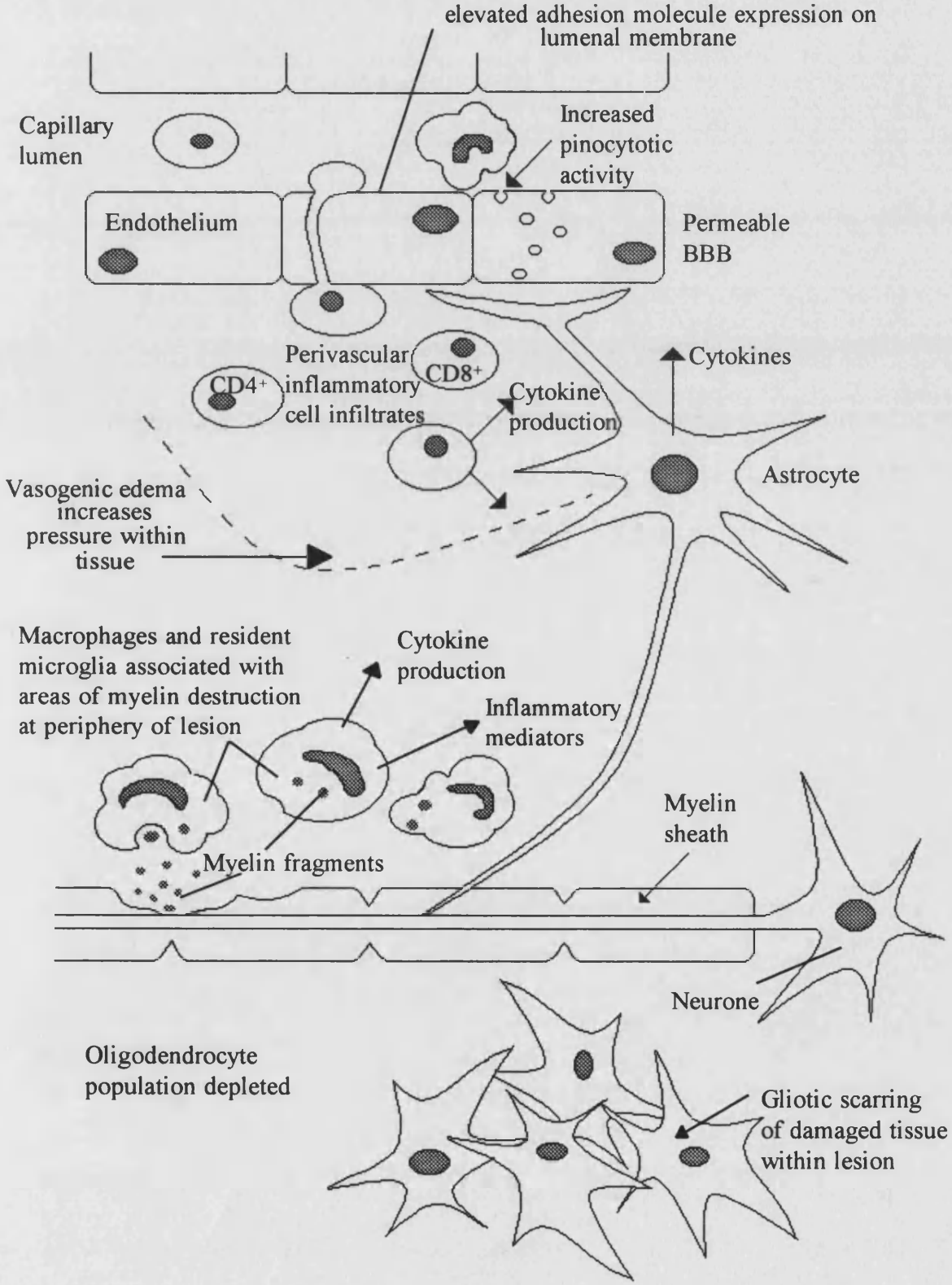
Ultimate confirmation of the condition can be gained at post-mortem when the CNS pathology of this demyelinating condition can be scrutinised. The disease

exclusively affects the white matter with a predilection for the periventricular, brainstem, spinal cord and optic nerve tissues. Early lesions develop around small blood vessels forming perivascular cuffs of inflammatory lymphocytes and macrophages surrounded by a vasogenic edema exudate. Myelin degradation is not associated with the initial perivascular pathology, but within the larger expanding lesions active demyelination of neuron sheaths is apparent. Myelin breakdown products appear in the surrounding parenchyma and within the cytoplasm of the CNS microglia and invading macrophages, which are implicated in the demyelination process. Axonal damage is minimal the majority remaining intact, however their conductive efficiency is dramatically impaired once myelin is lost. As the lesions coalesce they create large demyelinated areas termed plaques. Active plaques continue to spread through myelin degradation at their periphery while the tissue in the centre is characterised by a reduced or absent oligodendrocyte population and an intense astrocytic proliferation creating gliotic scarring (Figure 3). Some lesions may be observed to have undergone a degree of remyelination, but it is often incomplete and distinguished by abnormally short internodes. Greater detail of the CNS cellular pathology of MS may be found in the comprehensive reviews of (Prineas & Wright, 1978; McFarlin & McFarland, 1982b; McAlpines Multiple Sclerosis, 1985; Raine & Scheinberg, 1988; Adams, 1977; Cuzner *et. al.*, 1988).

The pathology exhibited at post-mortem can only extend our knowledge of the progression of this human disease. To study the development of disease, the underlying dysfunctional mechanisms and to research potential therapeutic strategies an experimental alternative is required. The model most frequently used for this purpose is experimental allergic encephalomyelitis (EAE) (Paterson, 1978; Raine, 1984).

Figure 3: Schematic Representation of MS Plaque Pathology

Neurovascular dysfunction and perivascular inflammation characterise the expanding margins of MS plaques. Ongoing demyelination is also associated with peripheral areas. Within plaques gross myelin loss, oligodendrocyte depletion and astroglial scarring is seen.



1.4 Experimental Allergic Encephalomyelitis

The model EAE is principally a T cell-mediated autoimmune disease affecting the CNS. The condition shares many similarities with MS, summarised in Table 1.

1.4.1 Model Development and Induction

The development of EAE arose from the studies instigated following reports of paralytic accidents associated with the Pasteur rabies vaccine termed 'postrabies immunisation encephalomyelitis' in the late nineteenth century. The disorder was thought to arise from contamination of the vaccine with remnants of the neurological tissue in which it was cultured. In the 1930's Rivers produced an acute disseminating encephalomyelitis in monkeys following repeated injections of normal rabbit CNS tissue and this represented the first work on EAE. Development of adjuvants in the 1940's allowed the evolution of faster developing, highly reproducible models of EAE following a single inoculation (Freund *et. al.*, 1947).

EAE can now be induced in a variety of species by modifying the combination of neurological encephalitogen and adjuvant in the emulsion. Immune cell activation occurs at the lymph nodes draining the subcutaneous injection sites and results in a range of clinical courses and tissue pathologies each dependant on the inoculum content and species susceptibility (Shaw & Alvord, 1984; Villas *et. al.*, 1991). Genetic susceptibility appears to be a prerequisite for successful manifestation of EAE, with both major histocompatibility complex and non-major histocompatibility complex-linked genes being implicated (Steinman, 1992; Tournier-Lasserve & Bach, 1993). The main encephalitogenic component of EAE inoculum has been identified as myelin basic protein (MBP) which when purified and administered in combination with complete Freund's adjuvant can also elicit EAE. MBP has been extensively studied resulting in the identification of a number of encephalitogenic peptide sequences of varying potency for different species and strains (Einstein *et. al.*, 1972;

Table 1: Comparison of the Pathological and Immunological characteristics of EAE and MS.

	EAE	MS
Autoantigen	MBP,PLP	?
Chronic relapsing course	+	+
Lesion Pathology:		
BBB breakdown	+	+
Edema formation	+	+
Predilection of lesions for white matter	+	+
Perivascular inflammatory infiltrates	+	+
$CD4^+$ T-cells	+	+
Macrophages	+	+
Meningitis	+	+
CNS demyelination	±	+
Axonal survival	+	±
Immune Responses:		
T-cell mediated disease	+	?
Linkage to MHC class II	+	+
Antibodies to CNS antigens in CSF	+	+
IgG deposition at lesion site	±	+
Circulating myelinotoxic antibodies	+	+

MHC, major histocompatability complex

Sources, Paterson 1978, Raine 1984

Brostoff, 1977). Other myelin components such as proteolipid protein and myelin oligodendroglia protein could all function as target antigens and have some success in EAE induction although they have been studied less extensively (Kuchroo *et. al.*, 1992; Linington *et. al.*, 1993).

1.4.2 Neurological Deficits and Pathology

Following sensitisation with an antigenic emulsion EAE neurologically manifests itself 10-14 days post-inoculation (PI). An overnight decrease in body weight is followed progressively over subsequent days by tail droop, weakness of the hindlimbs leading to paralysis and incontinence (Simmons *et. al.*, 1984). Some methods of EAE induction typically result in death, but in the acute monophasic Lewis rat model recovery ensues producing clinically well, weight gaining animals by day 20 PI. Recently chronic relapsing forms of the model have been developed in the guinea pig strain-13 and the Biozzi mouse, which mimic more closely the clinical course of MS (Raine & Stone, 1977; Baker *et. al.*, 1990).

Evidence that EAE is a CD4+ T cell mediated disease comes from adoptive transfer experiments where stimulated lymph node cells from sensitised animals injected into naive histocompatible recipients elicit a typical acute disease and pathology (Paterson, 1960; Stone, 1961; Pettinelli & McFarlin, 1981). Further confirmation has come from encephalitogenic T cell line and monoclonal antibody studies (Ben-Nun *et. al.*, 1981; Brostoff & Mason, 1984). The transfer of serum from sensitised animals into the circulation of uninoculated recipients does not effect the CNS, indicating that humoral factors do not initiate disease. Only by circumventing the blood-brain barrier can demyelination and mild EAE lesions be induced, although without neurological sign development (Jankowic *et. al.*, 1965; Lassman *et. al.*, 1981).

In common with the human counterpart, EAE displays perivenular foci of mononuclear cell inflammation within the white matter, with a predilection for the

spinal cord, brainstem and the ventricular area. The invading lymphocytes, macrophages and plasma cells are accompanied by a vasogenic plasma exudate and fibrin deposition (Levine *et. al.*, 1966; Lassman *et. al.*, 1986; Sobel *et. al.*, 1988). There is limited perivascular myelin damage noted in some models including the appearance of macrophages within the lesion site containing myelin debris, although this is a rare event in the acute monophasic condition. The chronic relapsing models display greater myelin degradation, which may be the result of a longer experimental time course. Lesions from these models display demyelination, remyelination, oligodendrocyte loss and glial scarring however the plaque development seen in MS is not duplicated (Raine, 1983; Ohkawa, 1989). Reactive gliosis may occur independently of any myelin damage such as is seen in the early pathology of the spinal cord in the Lewis rat acute model (Smith & Eng, 1988).

1.5 BBB Breakdown During MS and EAE

A pivotal step in the development of pathological and neurological signs in both MS and EAE is the focal disruption of the BBB prior to lesion development.

1.5.1 BBB Pathology in MS

The characteristic loss of barrier function in MS was initially observed using trypan blue. The dye was extravasated into CNS lesions corresponding histologically to the white matter plaques of the MS brains analysed after death (Broman, 1964). Recent advances in brain scanning have led to the development of MRI which has allowed visualisation of MS lesion patterns. Further information may be gained by enhancing the image using an intravenous injection of gadolinium-diethylene-triamine pentaacetic acid (Gd-DTPA). Gd-DTPA is a paramagnetic contrast agent which has the ability to cross the damaged BBB highlighting areas of vasogenic

edema entry and inflammation as confirmed by parallel ultrastructural examination of tissues (McDonald & Barnes, 1989). Enhancement of the image locates active sites, marking new lesion development or older plaque enlargement (Bastianello *et. al.*, 1988). Gd-DTPA enhancement is noticeably greater in clinically active patients and while there can be good correlation between presented signs and enhancing lesion geography, Gd-enhancement often highlights clinically silent lesions, even during remission (Grossman *et. al.*, 1988). BBB breakdown, as demonstrated by Gd-DTPA MRI, is a consistent early event and can be detected up to two weeks before conventional MRI abnormalities or clinical deficits are reported (McDonald & Barnes, 1989).

Neurological deficits appear to be linked to BBB breakdown and the subsequent inflammatory events. Indeed, cellular degradation of axonal myelin cannot alone account for the clinical course of MS as remyelination is unable to occur fast enough to explain the onset of remission (Grossman *et. al.*, 1987; Levine *et. al.*, 1966). Vasogenic exudation of plasma constituents into the CNS may independently create a variety of clinical and pathological effects. An increase in intercellular tissue volume due to edema formation may (i) constrict nerve fibres, (ii) disrupt the layering of the myelin plus its proximity to the axon and (iii) alter the ionic environment at the nodes of Ranvier, all impairing the efficiency of neural conduction. Furthermore, *in vitro* studies using CNS cell cultures have demonstrated the existence of humoral neuroelectric blocking factors and myelinotoxic components in the plasma of MS patients, which would prove detrimental following extravasation (Bornstein & Crain, 1965; Cerf & Carels, 1966; Raine *et. al.*, 1973; Schauf *et. al.*, 1978; Schauf *et. al.*, 1981; Wiley *et. al.*, 1992).

1.5.2 BBB Pathology in EAE

The characteristic loss of barrier function, as for MS, was initially described using trypan blue which was extravasated into CNS areas corresponding histologically

to inflammatory areas in EAE (Barlow, 1956). Radioactive tracer studies of BBB opening in EAE have demonstrated that increased neurovascular permeability to plasma protein occurs at the onset of neurological deficits (Rumjanek *et. al.*, 1984a; Leibowitz & Kennedy, 1972), although some investigators have suggested that leakage occurs just prior to development of the disease (Butter *et. al.*, 1991; Juhler *et. al.*, 1984). Indeed, tracer studies employing markers of small molecular weight such as ^{14}C-mannitol indicate that barrier properties are abnormal as early as day 7 PI (Lam, 1986). The pattern of inflammatory cell extravasation appears not to coincide directly with plasma solute entry. The main leukocyte influx is generally reported as occurring slightly later than initial BBB opening (Rumjanek *et. al.*, 1984a; Juhler *et. al.*, 1984). However, a number of histology reports document cellular entry into the CNS as early as day 5 PI (Traugott *et. al.*, 1982, 1985, 1996). A separation in time between BBB breakdown to solutes and the recruitment of inflammatory cells into the CNS parenchyma would therefore appear to be indicated for EAE.

Gd-DTPA enhancement of inflammatory lesions during EAE have demonstrated a linear relationship between lesion number and relapse and a direct link between the duration of enhancement and the duration of relapse (Hawkins *et. al.*, 1991). The connection between BBB dysfunction and the onset on neurological deficits is supported by observations from other experimental models. Tunicamycin-poisoned rats develop neurological signs typical of those seen in acute EAE. Using this model, Kerlero de Rosbo *et. al.* (1987) demonstrated elevated levels of albumin and IgG within the CNS of animals which occurred coincident with the onset of deficits, but no evidence of demyelination was observed. Furthermore, in an animal model of demyelination induced by cuprizone, no inflammation or increase in BBB permeability was found, demonstrating that cerebrovasculature disruption is not caused by demyelinating events (Bakker & Ludwin, 1987). Therefore while clinical deficits and neuroendothelial dysfunction appear related, neither seem dependent on demyelination.

1.5.3 Ultrastructural Aberrations

Histological examination of CNS tissue from EAE animals has revealed an increase in the number of pinocytotic vesicles evident in endothelial cells within active lesion sites (Claudio *et. al.*, 1989; Hawkins *et. al.*, 1992). Hawkins (1992) demonstrated that the raised vesicle production was energy-dependent as it could be suppressed by prior perfusion with the metabolic inhibitor 2,4-dinitrophenol. Additional support for a change in the metabolism of the endothelia was reported by Kato (1989) who showed that the Na^+-K^+-ATPase activity, which is normally confined to the abluminal membrane, was distributed both luminally and abluminally during the acute stage of chronic-relapsing EAE. Furthermore, during the inactive stage of this model no ATPase activity was apparent on either membrane indicating a different but continuing BBB impairment. Indeed, metabolic abnormalities may be further indicated by the decrease in the mitochondrial content of cerebrovascular endothelial cells, which at the height of disease are reduced to a level associated with non-neurovascular endothelia (Oldendorf *et. al.*, 1977; Claudio *et. al.*, 1989). Biopsy analysis of MS patient brains supports the observation of elevated vesicular activity in the neuroendothelia, but a corresponding decrease in mitochondrial number has not been validated (Brown, 1978).

Hawkins *et. al.* (1992) saw no evidence of tight junctional opening during their tracer studies, and following metabolic inhibition of the vesicular transport of lanthanide no deposition of tracer was discernible in the perivascular space. However, size-dependent extravasation of radioactive markers led Lam *et. al.* (1986) to suggest that if the barrier dysfunction was led by vesicular transport then the tracers should be deposited extravascularly at a similar rate. Therefore, a size-related disruption of the tight junctional complexes was implicated. Furthermore, impairment of BBB membrane integrity may have been indicated by the increased permeability induced in the tunicamycin-poisoning study of Kelero de Rosbo *et. al.* (1987) as the biological

activity of this molecule is connected with impaired synthesis of structural glycoproteins.

1.5.4 Mechanisms of BBB Perturbation

The biological trigger for these endothelial abnormalities is unknown. However, endogenous steroid levels are strongly linked with normal BBB control. Studies in normal rats and mice have demonstrated that administration of the synthetic glucocorticoid, dexamethasone, can decrease BBB permeability to labelled sucrose and large vascular tracer molecules (Hedley-Whyte & Hsu, 1986; Ziylan *et. al.*, 1988). Furthermore, adrenalectomy in normal rats induces an increased cerebrovascular extravasation of albumin which can be reversed by exogenous steroid administration (Long & Holaday, 1985). Interestingly, basal corticosterone levels appear to be an important element in strain-susceptibility to EAE (Mason *et. al.*, 1990). One aspect of steroid control is the modulation of prostaglandin and leukotriene synthesis (Weidenfeld *et. al.*, 1987). These molecules have been demonstrated to be important in inflammation and elevated levels of the potential barrier perturbators have been shown EAE (Bolton *et. al.*, 1984b; Prosiegel *et. al.*, 1989; Fretland *et. al.*, 1991). The coagulation cascade, which the endothelial cell has the capability to modulate, may be involved in either BBB opening or the ensuing inflammatory response. Perivascular fibrin deposition in EAE is associated with neurovascular permeability and symptom onset, both of which may be reduced by inhibition of the plasminogen activator (Koh & Paterson, 1987).

Barrier permeability may also be affected by a variety of biological mediators released by activated immunological cells. Mast cells release histamine, a potent vasodilator, during early or mild inflammation. Histamine levels have been reported to be raised at around days 11-13 PI in EAE and have been shown to increase pinocytotic vesicle activity in neural endothelial cells (Dux & Joó, 1982; Orr & Stanley, 1989). Inflammatory lymphocytes and macrophages release a variety of

cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-1, both of which are raised in the CNS of EAE animals and have been shown to possess permeability-inducing properties *in vitro* (Henning *et. al.*, 1987; Brett *et. al.*, 1989; Baker *et. al.*, 1991).

Evidence indicating which mechanisms are involved in the breakdown of normal neurovascular function is limited at this time. However, alteration of normal BBB permeability is clearly an obligatory step in the appearance of clinical signs and the development of lesions in MS and its animal counterpart EAE. Therefore, correction of pathological barrier disruption could both improve clinical presentation and limit lesion development.

1.6 Treatment of MS

Therapeutic goals in the treatment of MS vary according to disease development. In relapsing-remitting MS the aims of therapy are: (1) to improve the patients rate of recovery from relapse; (2) to decrease the incidence of future relapses; (3) to reduce the accumulation of additional disabilities; and (4) to prevent the development of a chronic progressive course of disease. In patients who already display chronic progressive MS the primary objective is to halt the deleterious progression of the condition.

Current treatment of MS patients both to reduce the severity of clinical episodes and to limit long-term deterioration is far from satisfactory. The natural history of the disease is highly unpredictable due to the relapsing-remitting sequence of events and the additional possibility of chronicity or stabilisation developing at any time. Therefore, MS is one of the most difficult diseases in which to effectively judge the response to therapy (Kappos, 1988). An increase in the last decade of well-controlled and designed drug trials has provided greater definitive proof of the

efficacy of individual pharmacological agents in this condition (Goodin, 1991; Ebers, 1994). Furthermore, the extension of drug trials to include an MRI appraisal of pharmacological control of inflammatory lesion pathology will prove beneficial (Grossman *et. al.*, 1988; Willoughby *et. al.*, 1989; Kermode *et. al.*, 1990; Ebers, 1994).

Therapy of the human demyelinating condition has for many years been based upon the anti-inflammatory and immunosuppressive effects of steroids and the global immunosuppressive actions of drugs such as azathioprine. Recently, alternative strategies have been examined including immunomodulation with compounds such as cyclosporin A and a variety of cytokines plus targeting of T cell subsets with monoclonal antibodies and induction of peptide tolerance (Dijkstra, 1993; Ebers, 1994). While many therapeutic regimes have been applied to the treatment of MS only the major forms of treatment and emerging therapies will be considered in the following sections.

1.6.1 Current Drug Treatments

Adrenocorticotrophic hormone (ACTH) and adrenal corticosteroids are general anti-inflammatory agents as well as having immunosuppressive properties. Intramuscular ACTH and oral corticosteroid therapies have been favoured in MS for many years, with moderate doses generally being shown to increase the rate of recovery from relapse (Rose *et. al.*, 1970; Troiano *et. al.*, 1987; Milanese *et. al.*, 1989). However, the long-term prognosis for MS does not appear to be favourably altered by steroidal intervention. Hoogstraten & Minderhound (1990) concluded that the initial benefit gained from 100 units ACTH tapered over 12 weeks was not evident at six months and that a higher relapse rate may even have been indicated. Recently, high dose intravenous methylprednisolone treatment of acute clinical episodes has found popularity. Typically, doses of 500-1500mg methylprednisolone per day are given over 3-7 days with a tapering dose of oral prednisone from 60-0mg

over 10 days (Dowling *et. al.*, 1980; Milligan *et. al.*, 1987; Lyons *et. al.*, 1988). Barnes *et. al.* (1985) report a more rapid improvement with this regime than that achieved with ACTH but finds no long-term benefit, while Milligan and workers (1987) found favourable clinical effects for both relapsing and chronic progressive patients.

Corticosteroid action is not limited to modulation of pathological events and suppression of the immune system. Steroids are biologically active throughout the body participating in the regulation of many normal processes. Therefore, chronic treatment with either ACTH or glucocorticoids may be associated with a variety of unwanted side-effects. Although high-dose intravenous methylprednisolone is reportedly free from serious adverse affects and generally produces fewer unwanted sequelae than ACTH, uncomfortable reactions are routinely experienced (Compston, 1988; Miller *et. al.*, 1992).

The active recruitment of non-specific inflammatory T cells and macrophages into the CNS of MS patients has led to many immunosuppressive or immunomodulatory drug trials. Azathioprine, which modifies nucleic acid or protein metabolism of cells, has the longest history of use in MS, but like many drugs has not always been studied in the context of well controlled and blinded trials. A controlled study by Ellison (1989) followed chronic progressive patients treated with azathioprine for three years. The resultant relapse rate was concluded to have been halved, but the adverse side-effects, which were increased by combined therapy with steroids, were found to out-weigh any beneficial results.

Cyclophosphamide, an alkylating agent which interferes with nucleic acid metabolism, is toxic to lymphocytes when used at a high concentration. The drug has shown variable success in the treatment of chronic progressive patients but the threat of long-term toxic effects has precluded its general use (Mauch *et. al.*, 1989; Likosky *et. al.*, 1991). Similarly, cyclosporin A (CSA), which down-regulates IL-2 production by T cells, showed moderate clinical improvement in trials when administered at 7-

7.5mg/Kg body weight. However, the high incidence of hypertension, renal insufficiency and anaemia associated with the treatment prevented the proposal of further trials at higher doses (Rudge *et. al.*, 1989; Ruutiainen *et. al.*, 1991).

1.6.2 Emerging Drug Treatments

Monoclonal antibodies can target individual proteins which may mediate disease or be intimately involved in unwanted immunological responses including specific T cell surface receptors such as CD3, CD4, and CD25. Trials with anti-CD3 antibodies were found to successfully deplete the appropriate T cell subsets in MS patients, but unacceptable side-effects including fever and hypertension, were also encountered (Weiner & Paty, 1989). The human immune response to the non-self murine monoclonal antibodies was believed to be the major cause of trial failure and less antigenic chimeric antibodies are currently being developed for use in future therapeutic trials.

Manipulation of the endogenous cytokine levels during disease is an area currently under investigation. Interferons (IFN), produced naturally by the body, possess immune-regulatory properties yet deficient levels are found in the circulation of the majority of MS patients. IFN-α and γ proved ineffective or detrimental in tests while initial clinical trials of IFN-β were contradictory in their findings and used statistically limiting numbers of patients (Jacobs *et. al.*, 1986; Baumhefner *et. al.*, 1987; Huber *et. al.*, 1988; Milanese *et. al.*, 1990). A large well-controlled multicentre study of IFN-β therapy recently reported a successful reduction in exacerbation rates in relapsing-remitting patients (IFNB MS Study Group, 1993). Similar success has not been indicated for chronic-progressive patients and the long-term effects of IFN-β have yet to be identified.

Finally, Copolymer-1, a synthetic peptide originally designed as an MBP analogue, was found to suppress the development of autoimmune disease and is now a leading compound in the development of peptide tolerance agents for use in MS. A

double-blind pilot trial of the peptide in early relapsing-remitting patients found a favourable reduction in the number of relapses (Bornstein *et. al.*, 1987).

1.6.3 Correction of BBB Dysfunction in MS

Clinical deficits in MS are clearly linked to abnormal BBB function, therefore control of cellular infiltration and vasogenic edema would appear an obvious therapeutic strategy (Grossman *et. al.*, 1988; Hawkins *et. al.*, 1990; Hawkins *et. al.*, 1991). However, few drug studies other than those employing high dose steroids have analysed pharmacological efficacy at the level of the neurovasculature.

Evidence of glucocorticoid action at the BBB in MS comes from serial Gd-enhanced MRI images of MS patients. MRI analysis during and after high-dose steroid therapy has shown a reduction in enhancing image size following treatment, indicative of decreased barrier dysfunction, which correlated well with clinical improvement (Barkhof *et. al.*, 1991; Burnham *et. al.*, 1991). Conversely, no reported improvements were seen by Kesselring *et. al.* (1989) in enhanced MRI scans at 15 days post-treatment. However, a more recent MRI study by Miller *et.al.* (1992) demonstrated that rapid improvement of BBB abnormalities were seen with steroid therapy but were not maintained as brain lesions re-enhanced within days of ending treatment although clinical remission persisted. Therefore while steroid treatment in response to clinical exacerbations may be appropriate, a role beyond abatement of symptoms is not demonstrated.

The recent multicentre trial of IFN-β therapy reported concurrent MRI analysis alongside traditional evaluation of exacerbation rate (IFNB MS Study Group, 1993; Paty *et. al.*, 1993). The study demonstrated a significant reduction in active lesion presentation over the course of a year in the treated groups, indicating diminished inflammatory activity possibly as a result of improved cerebrovascular function (Paty *et. al.*, 1993).

1.6.4 Targeting the BBB in EAE

Investigaton of pharmacological agents in EAE enable correction of abnormal barrier permeability to be quantitatively analysed using a range of radioactively labelled markers tailored to the needs of the study. Nevertheless, few drugs have been analysed with correction of aberrant cerebrovascular function in mind. An isolated study by Reiber and Suckling (1986) found disease suppression and normal BBB permeability to proteins following prophylactic administration of CSA in EAE-sensitised guinea-pigs. However, determination of the effects of therapeutically administered CSA was not made.

EAE provides a unique opportunity to study the ongoing aberrant mechanisms present in immune-mediated neurological disease and to employ pharmacological agents in identifying the underlying abnormalities. Such an approach has been used to understand the mechanisms which lead to BBB breakdown in studies on the catecholamines noradrenalin and adrenalin, which have been shown to influence the development of EAE (Wesselman, 1987; Mackenzie *et. al.*, 1989; Leonard *et. al.*, 1991). The specific involvement of the adrenergic receptor was indicated in studies by Brosnan *et. al.* (1985) who sucessfully employed the α_1 receptor antagonist prazosin in suppression of acute EAE. Goldmuntz *et.al.* (1986) extended the analysis of prazosin in EAE examining cerebrovascular extravasation of radiolabelled albumin. BBB function was significantly improved and cellular infiltration into CNS tissue was delayed following antagonism of the α_1 - adrenergic receptor.

Non-immune models of neurovascular disruption have highlighted enzyme cascades and receptors potentially involved in barrier breakdown (Koenig *et. al.*, 1992; Ohnishi *et. al.*, 1992; Sharma *et. al.*, 1992). Examination of the suggested causes of BBB abnormalities, which include polyamine synthesis, arachidonic acid metabolism, H_2-histamine receptor stimulation and NMDA-receptor activity, may lead to a new understanding of the development and suppression of EAE. Antagonists of enzyme and receptor activity such as ifenprodil, MK801 (NMDA-receptor

antagonists), α-difluoromethylornithine (inhibits polyamine synthesis), BW755C (lipooxygenase inhibitor) and cimetidine (H_2-receptor antagonist), could therefore be employed as pharmacological tools to uncover abberrant mechanisms in neuroantigen-induced BBB breakdown.

Clearly there remains no satisfactory therapy for MS patients. Existing treatments carry unacceptable side-effects and / or demonstrate beneficial outcomes in only a small percentage of the treated MS patients. In general, the actions of therapies may be too broad-based, although recent developments in treatment are showing increasing specificity. Section 1.5 highlighted the primary importance of BBB breakdown in both MS and the experimental condition EAE and considered the close association between neurovascular dysfunction and the expression of neurological deficits. Analysis of therapeutic agents and design of new pharmacological strategies has however largely ignored this aspect of neurological disease

1.7 Aims of the Project

(i) Identification and development of a technique with sufficient sensitivity to quantitate BBB dysfunction during EAE and to detect beneficial improvements following drug administration.

(ii) Characterisation of glucocorticoid control of neurovascular function during EAE.

(iii) Assessment of the neurovascular regulatory capabilities of drugs shown to limit the severity and duration of neurological signs in EAE.

(iv) Investigation of abnormal mechanisms underlying the development and maintenance of BBB pathology during EAE.

2.
Materials and Methods

2.1 Animals

Inbred male Lewis rats, weighing 200-250g, were selected from either stock bred on site or animals purchased from Bantin and Kingman Ltd. Wistar rats were bred on site and used at approximately 300g body weight. Experimental rats were housed 4-6 per cage on wood flakes [Cardiff Sawdust Co.], with food [SDS, diet C.R.M.] and water available *ad libitum*.

Spinal cord neuroantigen for EAE-inoculation was obtained from both male and female Dunkin-Hartley guinea-pigs of body weight >600g, either bred on site or by Bantin and Kingman Ltd.

2.2 Inoculation for EAE

The acute Lewis rat model of EAE was induced using whole guinea-pig spinal cord as the source of neuroantigenic material. The spinal tissue of Dunkin-Hartley guinea-pigs killed by CO_2 asphyxiation was exposed and removed from the vertebral canal without meningeal or peripheral nerve attachments. The tissue was washed in phosphate buffered saline (PBS) [Sigma] and blotted prior to storage at -20°C.

Encephalitogenic inoculum was freshly prepared on the day of sensitisation. The spinal tissue was finely cut and homogenised in sterile PBS (1ml/1g tissue) using a 1ml syringe. Incomplete Freund's adjuvant [Difco Laboratories] was introduced at 1ml/g whole tissue and supplemented with *Mycobacterium tuberculosis* $H_{37}Ra$ [Difco Laboratories] at a concentration of 20mg/ml adjuvant. The mixture was vigorously shaken prior to repeated aspiration between two connected syringes. Finally, the cohesive properties of the inoculum were tested on cold water. Using a 19 gauge x 1.5" needle, 1ml syringes were filled with 0.45ml inoculum and fitted with a 23 gauge needle. Unsedated male Lewis rats received 0.1ml inoculum in each hindfoot pad.

Control animals received 0.1ml per hindfoot pad of a complete Freund's adjuvant (CFA) emulsion. The CFA-inoculum was prepared as for EAE-inoculum but with 1ml PBS substituted for every 1g guinea-pig spinal cord used. The cohesive properties of the final emulsion were again tested on water.

2.3 Clinical Assessment of EAE

Animal body weight was monitored daily throughout the course of study beginning on the day of inoculation - day 0 PI (D0P.I.). Weight loss in the acute phase of EAE was expressed as the percentage change in body weight between peak weight prior to disease onset and weight on the day of sampling.

Neurological signs were scored daily subsequent to observed weight loss, as follows: 0.5 = loss of tone at the tip of the tail : 1 = flaccid tail (FT); 2 = ataxic gait and hindlimb hypotonia (HLW); 2.5 = severe hindlimb weakness, unable to support own body weight; 3 = partial paralysis of hindlimbs (HLPP); 4 = complete hindlimb paralysis with associated urinary and faecal impaction incontinence(CHLP). Symptoms observed for a treatment group on the day of sampling were expressed as a mean neurological score (MNS).

Generally EAE-inoculated animals demonstrated weight loss approximately D10-11PI and displayed clinical signs for approximately 6 days thereafter, this was termed the 'Acute' phase. At D20 ± 2 PI previously affected animals, now free of neurological deficits, had resumed weight gain and were said to be in 'Early Recovery'. A 'Late Recovery' time point was selected for study at D35PI.

CFA-inoculated control animals showed no neurological deficits and continued to gain weight throughout the study periods. However, adjuvant arthritis became apparent in rats when the length of investigation was extended to study timepoints PI equivalent to the 'Recovery' phases in EAE-sensitised animals.

2.4 Iodination of Rat Serum Albumin (RSA) With ^{125}Iodine (^{125}I)

Iodination of RSA [Sigma] with ^{125}I was undertaken using the McConahey & Dixon modification of the chloramine-T method originally described by Greenwood, Hunter & Glover (1963).

An equal volume of 100mM HCl was added to 1mCi ^{125}I [NEN: diluted in 10^{-5}M NaOH] followed by 10mg RSA dissolved in 200µl sterile PBS. Initiation of the radioiodination was achieved by addition of freshly prepared chloramine-T [Fisons, SLR grade: 10mg/ml sterile PBS] at a ratio of 10µg/mg RSA. Following mixing, the radioiodination was left to proceed for 10min after which the reaction was terminated by addition of 20µl of freshly prepared sodium metabisulphite [BDH] / potassium iodide solution [Fisons, SLR grade][125mg/250mg in 10ml sterile PBS].

The unreacted radioiodine was removed by exclusion chromatography on a PD-10 column [Pharmacia] containing Sephadex G25. Separation by this method is size-dependent whereby large molecules, such as albumin, are excluded from the matrix pores and move rapidly through the interstitial spaces unlike smaller molecules which move at a slower pace passing within the pore spaces. The eluent was collected in 1ml fractions and 5µl aliquots screened on an LKB gammamaster. Those fractions demonstrated to contain the iodinated albumin peak were pooled and stored at 4°C.

Activity of the iodinated product was determined by analysing dilutions of the stock in duplicate on an LKB Gammamaster [efficiency 70%] and processing the results using equation ①.

$$\frac{(\text{cpm} \times \text{dilution factor}) \times 100}{70} = \text{dpm/ml} \qquad ①$$

Where: cpm = counts per minute;

dpm = disintegrations per minute;

2.23×10^6 dpm = 1µCi.

Purity of the radiolabelled RSA was determined by addition of 20µl 20% trichloroacetic acid to an equal volume of the ^{125}I-RSA stock. After 15min at room temperature the precipitated protein was removed by centrifugation at 10 000g and the activity remaining in the supernatant measured [LKB Gammamaster]. Typically, contamination of the iodinated product with unbound iodine did not exceed 7%.

2.5 Blood Volume Markers

2.5.1 ^{113m}Indium (^{113m}In) and ^{111}Indium (^{111}In) Labelling of Transferrin

The eluate from a sterile ^{113m}In generator [Amersham] or an aliquot of ^{111}In-chloride [NEN] was added to rat plasma and gently mixed to bind the isotope to transferrin (Adatepe *et. al.*, 1969; Hosain *et. al.*, 1969). The final activity of ^{113m}In and ^{111}In radiolabelled plasma was 200µCi/ml and 20µCi/ml respectively. Both were administered i.v. in the tail vein using a 25 gauge needle, at a volume of 0.5ml per animal.

The percentage of isotope bound to plasma protein was determined by addition of an equal volume of 20% trichloroacetic acid to an aliquot of radiolabelled plasma. After 15min at room temperature the precipitated protein was separated by centrifugation at 10 000g and the activity of supernatant and pellet fractions counted on an LKB minigamma counter.

2.5.2 ^{111}In-tropolonate Labelling of Red Blood Cells (RBC)

The method was developed from the studies of Osman and Danpure (1987). Blood from adult Wistar rats was collected by cardiac puncture in citrate-phosphate-dextrose [Sigma], and centrifuged at 300g for 10mins. The plasma / white blood cell layer was collected and respun at 1100g for 15mins to generate cell-free plasma. The RBC layer was resuspended in Hepes saline buffer [20mM Hepes, Gibco; 0.8% NaCl]

and washed three times at 300g to remove leukocytes, resuspending at a final concentration of 5 x 10^8 cells/ml. An addition of 100µl/ml ^{111}In-tropolonate [4 x 10^{-3}M tropolone containing 20µCi ^{111}In (NEN) per millilitre] for every 5 x 10^8 RBC was made, followed by incubation of the mixture at 37°C for 20mins. The labelled cells were washed three times and resuspended at 5 x 10^9 cells / 0.5ml cell-free plasma for *in vivo* use. Analysis of 10µl from each post-labelling wash and final radiolabelled cell preparation was routinely performed to confirm that adequate labelling had taken place.

2.6 Quantitation of Blood-brain barrier (BBB) Permeability

A variety of methods were explored to find a reliable technique which quantitated a large window of difference between normal and disease BBB permeabilities suitable for analysis of potentially corrective pharmacological agents. Section 2.6.1 lists all techniques tested for the measurement of edematous water accumulation in the CNS. However, section 2.6.2 details only the final method developed for the quantitation of BBB permeability to albumin which was subsequently used to analyse pharmacological restoration of neurovascular function. During development of the technique (2.6.2) a variety of radiolabelled tracers and circulation times had been considered for the blood volume determination. While these variations in methodology are not documented in the materials and methods they are reported in full in Chapter 3.

2.6.1 Edema Measurement

Three methods were considered for the quantitation of edematous water extravasated into inflammatory areas of the CNS.

2.6.1.1 Dual Label Determination of Water Extravasation

To compare water and albumin extravasation within the same animal 10μCi tritiated water (^3H_2O), diluted in sterile PBS, was injected i.v. in the tail with a 25 gauge needle under halothane [RMB Animal Health Ltd.]/O_2 anaesthetic, 20-24 hours after ^{125}I-RSA administration. Following a 30min circulation period a cardiac blood sample was collected from the anaesthetised animal into a heparin-coated tube [Sterillin] and the rat killed with a lethal dose of euthatol [RMB Animal Health Ltd.]. Cerebellum (C), medulla-pons (MP) and cervical spinal cord (CSp) tissues plus blood samples were stored at -20°C until use. Samples were solubilised with 1ml Optisolve [LKB]/200mg wet tissue over 2-3 days with continuous shaking. After the addition of 10mls scintillant [Optiphase Safe, LKB] samples were analysed using a dual count parameter on an LKB Rackbeta 1219. Blood equivalent values were calculated as per equation ②.

$$\frac{\text{cpm / g Tissue}}{\text{cpm / ml Blood}} \times 100 = \text{BE} \qquad ②$$

2.6.1.2 Ethanol Extraction of ^3H_2O

The method was based on that detailed by Simmons *et. al.* (1982). Rats received 10μCi ^3H_2O diluted in sterile PBS (pH 7.2) i.v., which circulated for 30mins. Under halothane/O_2 anaesthetic a blood sample was collected by cardiac puncture using a 21 gauge needle followed by administration of a lethal dose of euthatal. The whole blood was stored in heparin-coated tubes. Weighed C, MP, CSp, thoracic spinal cord (TSp) and lumbar spinal cord (LSp) tissues were placed in 4mls analytical grade ethanol and agitated overnight at room temperature. Analysis of 1ml aliquots of ethanol-extracts and 0.1ml aliquots of plasma samples was performed on an LKB Rackbeta 1215 following the addition of 10mls scintillant.

Results were expressed as plasma equivalents (PE) (Equation ③):

$$\frac{^{3}\text{H cpm / g tissue}}{^{3}\text{H cpm / ml plasma}} \times 100 = {}^{3}\text{H PE} \qquad ③$$

2.6.1.3 Tissue Weight

Increased water content of the CNS parenchyma due to the influx of inflammatory edema may be quantitated by calculating the difference between wet and dry weights (Levine *et. al.* 1966,1977). Tissue sections C, MP, CSp, TSp and LSp were dissected, weighed and placed in an oven at 80°C. Once a constant weight was achieved a final dry weight was recorded and the % water content determined equation ④;

$$\%\ \text{Water Content} = \frac{\text{Wet weight (g)} - \text{Dry weight (g)}}{\text{Wet weight (g)}} \times 100 \qquad ④$$

2.6.2 Albumin Extravasation

Permeability of the cerebral vasculature was determined by quantifying the extravasation of radiolabelled RSA into CNS tissue, using a variation of the double radioisotope technique first described by Leibowitz and Kennedy (1972). The method employed a second isotope to correct for the ^{125}I-albumin circulating within the vasculature of tissues analysed. Animals were routinely sampled between 2-4pm to limit variation in results due to fluctuating endogenous glucocorticoid levels.

Rats received 10μCi ^{125}I-RSA diluted in sterile PBS i.v. in the tail, using a 25 gauge needle, while under halothane/O_2 anaesthetic. A day later 5 x 10^9 ^{111}In-RBC were injected by the same route under halothane/O_2, the animals remaining anaesthetised thereafter. Following 4.5mins circulation of the second isotope, a cardiac blood sample was collected using a 21 gauge x 5/8" needle and held in a

heparin-coated tube. A lethal injection of 0.1ml euthatal administered direct to the heart followed immediately. C, MP, and CSp tissues were dissected, washed in PBS, blotted and weighed. The amount of ^{111}In radioactivity present in each tissue and in 100µl blood aliquots was recorded on the open channel of an LKB minigamma counter [settings = 100-450kev]. Samples were stored at -20°C and recounted 3 weeks later on the ^{125}I channel [settings = 20-80kev] following ^{111}In decay. The individual isotope counts for each tissue were expressed in arbitrary units termed 'blood equivalents (BE)'.

$$\frac{\text{cpm / g Tissue}}{\text{cpm / ml Blood}} \times 100 = \text{BE} \qquad ⑤$$

The extravascular blood equivalent (EVBE), a measure of the radiolabelled albumin which has crossed the BBB and accumulated within the CNS, was calculated by equation ⑥.

$$^{125}I\ BE - {}^{111}In\ BE = EVBE \qquad ⑥$$

2.7 Drug Preparation and Administration

2.7.1 Dexamethasone (DEX)

Dexamethasone sodium phosphate [4mg/ml, DBL, UK] was diluted in sterile PBS and administered subcutaneously with a 25 gauge needle. Administered doses ranged from 0.01-1mg/kg body weight. DEX was injected twice daily for 2 days starting on the day of weight loss in the acute phase and following the loss of neurological signs and two consecutive days of weight gain during the early recovery phase. Animals were sampled on the day following the conclusion of treatment.

Vehicle-control groups received equivalent volumes of sterile PBS.

2.7.2 Cyclosporin A (CSA)

CSA [supplied by, Sandoz Laboratories, Switzerland] was dissolved at a concentration of 25mg/ml in olive oil at 60°C and administered orally with an oral dosing needle. Drug treatments ranged from 25-75mg/kg body weight and were administered once daily for 2 days starting on the day of weight loss in the acute phase and following the loss of neurological signs and two consecutive days of weight gain during the early recovery phase. Animals were sampled on the day following the conclusion of treatment.

Vehicle-control groups received equivalent volumes of olive oil alone.

2.7.3 FK506 (Tacrolimus)

FK506 [supplied by, Fujisawa Pharmaceutical Co. Ltd., Japan] was initially prepared according to previously published work in rats as a suspension in sterile PBS and administered orally using an oral dosing needle (Nalesnik *et. al.*, 1987; Venkataramanan *et. al.*, 1987; Inamura *et. al.*, 1988). Following receipt of a recommended protocol from Fujisawa, subsequent preparations of FK506 were dissolved in ethanol at 10mg/ml and diluted to 1mg/ml with sterile PBS 2% Tween 80 for intraperitoneal (i.p.) administration with a 25 gauge needle. Two concentrations of drug were studied: the 5mg/kg body weight dose was prepared for oral dosing; and the 10mg/kg body weight dose was prepared for i.p. injection. Treatment with both preparations was once daily for 2 days beginning on the day of weight loss in the acute phase and following the loss of neurological signs and two consecutive days of weight gain during the early recovery phase. Animals were sampled on the day following the conclusion of treatment.

Vehicle-control groups received equivalent volumes of vehicle preparations alone.

2.7.4 MK801 (Dizocilpine maleate)

MK801 [supplied by, Dr. L.L. Iversen, Merck, Sharp and Dohme Research Laboratories, Harlow, England] was dissolved for use in sterile PBS and injected i.p. at a concentration within the range 0.15-0.6mg/kg body weight. The prophylactic schedule required administration of MK801 once daily from D7-12PI with sampling of treatment groups on D12PI. During the acute phase therapeutic treatment was given once daily for 3 days starting on the day of weight loss and concluding on the day of sampling.

Vehicle-control animals received equivalent volumes of sterile PBS alone.

2.7.5 RU38486 (Mifepristone)

RU38486 [supplied by, Dr. R. Deraedt, Roussel UCLAF, France] was suspended in 1% carboxymethylcellulose [BDH] containing 0.05% Tween 80 and administered using an oral dosing needle at 20 or 40mg/kg body weight. Dosing was twice daily D9-11PI with a single dose on D12PI. Doses on D10 and 11PI were administered 1hr prior to DEX or DEX-vehicle (PBS) treatment. Animals were sampled on D12PI.

Vehicle-controls received equivalent volumes of vehicle alone.

2.8 Histology

To determine CNS tissue lesion load CSp tissues were dissected and stored in 10% formyl saline. Histological processing was performed in collaboration with the staff at the Department of Cellular Pathology, Royal United Hospital, Bath. Tissues were transferred to 50% cedarwood oil in molten paraffin wax for 30min at 60°C after which 6 changes of molten paraffin wax of 1hr duration were made. Finally, the sample was embedded in wax moulds and once hardened longitudinal horizontal

sections, 6μm thick, were cut at a standard depth defined as the widest point in the tissue.

Sections were mounted onto glass slides and stained with haemotoxylin and eosin as follows. Samples were immersed twice in xylene for 2min and then briefly in 50% xylene in alcohol, absolute alcohol, 95% alcohol, 70% alcohol, 40% alcohol and distilled water, each for 15sec. Sections were then stained with Harris haemotoxylin (without acetic acid) for 15min followed by a distilled water rinse and brief exposure to 0.1M HCl in 70% alcohol. Slides were counterstained with 1% eosin (yellowish) and returned to xylene from alcohol through graded solutions.

The number of perivascular infiltrates per section were determined under low power by light microscopy.

2.9 Corticosterone Radioimmunoassay (RIA)

Endogenous corticosterone levels in rat plasma samples were determined using a ^{125}I-corticosterone RIA [IDS]. Plasma samples had been prepared by centrifugation of heparinised whole blood at 300g and stored at -20°C until use. The RIA uses a limited amount of corticosterone antiserum for which the ^{125}I-labelled corticosterone and the endogenous steroid within the test sample compete. The percentage of bound radiolabelled antigen therefore decreases as a function of the increasing concentration of unlabelled endogenous antigen present in the test sample. The bound ^{125}I-corticosterone is separated from the unbound by precipitation using a second antibody directed against the original antiserum.

The RIA has a range of 0.5 - 62.5ng/ml and was performed according to the manufacture's instructions with all standards and samples assayed in duplicate. Plasma was diluted 1 in 10 for analysis. To each 0.1ml standard or sample, 0.1ml ^{125}I-corticosterone and 0.1ml anti-corticosterone were added, vortexed and left to incubate overnight at 4°C. A 0.1ml aliquot of the second precipitating antibody was

incorporated and the tubes incubated for 1hr at room temperature. Following the addition of 1ml of saline, tubes were centrifuged at 1500g for 15min at 4°C. The supernatant was decanted and the resultant pellet counted on an LKB 1277 Gammamaster for 2min. Non-specific binding (NSB) controls, from which the corticosterone antiserum was omitted, were assayed alongside standards and samples.

The percentage of bound ^{125}I-corticosterone (%B/B0) was calculated using equation ⑦. The unknown sample corticosterone values were then derived from the standard curve of %B/B0 against standard concentration (ng/ml) on semi-log graph paper. A representative standard curve is shown in Figure 4.

$$\%B/B0 = \frac{\bar{x}\ \text{cpm (s)} - \bar{x}\ \text{cpm (NSB)}}{\bar{x}\ \text{cpm B0} - \bar{x}\ \text{cpm (NSB)}} \times 100 \qquad ⑦$$

Where: $\%B/B0$ = % ^{125}I-corticosterone bound

$\bar{x}$ cpm (s) = mean cpm of sample or standard

$\bar{x}$ cpm (NSB) = mean cpm of NSB control

$\bar{x}$ cpm B0 = mean cpm of 0ng / ml standard (= 100% binding)

2.10 Creatinine Determination

Creatinine levels in plasma samples were determined using a quantitative colourmetric diagnostics kit [Sigma]. Plasma samples had been prepared by centrifugation of heparinised whole blood at 300g and stored at -20°C until use. To 0.3ml of water (blank), 3mg/dL standard, or sample, 3ml of alkaline picrate solution was added, mixed and allowed to stand at room temperature for 8-12mins. Using the blank as the reference solution, absorbances were read at 500nm (A') on a PU8610 spectrophotometer. Addition of 0.1ml acid reagent was made to all cuvets which were inverted and allowed to stand for 5mins at room temperature. Solutions were then reread at 500nm (A") and creatinine concentrations calculated from equation ⑧.

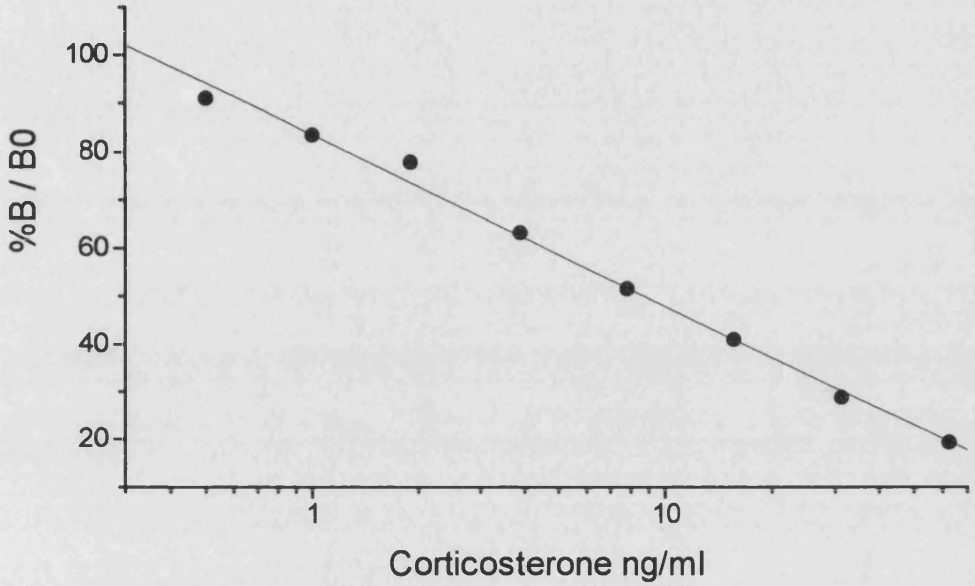


Figure 4: ^{125}I-Corticosterone Standard Curve

A typical standard curve achieved using a ^{125}I-corticosterone RIA [IDS]. The percentage of bound ^{125}I-corticosterone (%B/B0) decreases as endogenous (unlabelled) corticosterone increases.

$$\text{Creatinine (mg/dL)} = \frac{\text{Initial sample A}' - \text{Final sample A}''}{\text{Initial standard A}' - \text{Final standard A}''} \times 3 \qquad (8)$$

To determine the limit of linearity of the procedure for the cuvet size and instrument employed, a range of standards (0-10mg/dL) were tested as directed in the manufacturer's instructions. The linearity limit achieved for the PU8610 spectrophotometer is shown in Figure 5.

Values obtained for plasma creatinine concentration were compared to the normal range for rat plasma, published in the Clinical Biochemistry of Domestic animals (1989).

2.11 Statistics

Results were initially analysed for homogeneity of variance using Levene's test which assesses data from continuous, but not necessarily normal, distributions. Data not demonstrating equal variance ($p \leq 0.05$), was either log transformed and reassessed, or evaluated with the Mann-Whitney U test for non-parametric data, employing the Bonferroni correction factor when more than two groups were compared. Data groups with equal variances were analysed using the T-test for single comparisons or a one-way analysis of variance (ANOVA) for multiple comparisons. Following the recognition of differences by ANOVA, comparisons were made either throughout all groups by Tukey's pairwise assessment, or to a single control by Dunnett's test. Log-transformed data was tested for significant linear regression and the findings expressed as 'p' values and %fit, which indicates how close the actual values are to the fitted regression plot.

All statistical operations were performed using the computer program 'Minitab for Windows' and differences were assessed to be significant when $p \leq 0.05$. Results are expressed as mean values ± one standard deviation (SD) unless otherwise stated.

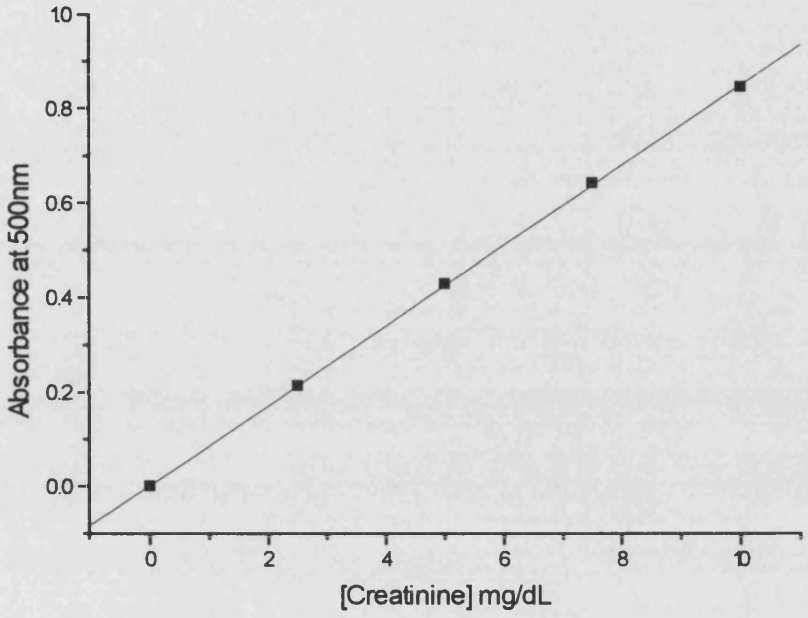


Figure 5: Standardisation of equipment for creatinine measurement. The limit of linearity was determined for the PU8610 spectrophotometer using macrocuvets. All subsequent measurements of creatinine concentrations within the range 0-10mg/dL would lie within the linear limits of the instrument.

3.
Development of an *In Vivo* Technique to Quantify BBB Permeability

3.1 Results

3.1.1 Protein Extravasation

A double radioisotope method for the determination of BBB permeability was chosen based on the original technique described by Leibowitz & Kennedy (1972) and subsequent modifications by Oldendorf & Towner (1974). The technique employs the neurovascular permeability tracer ^{125}I-labelled albumin, which circulates for up to 24 hours, crossing the barrier and accumulating at abnormal levels in regions of microvascular dysfunction. A second radiolabelled tracer is then injected systemically and allowed to equilibrate throughout the blood for up to 5 mins. This short time precludes significant extravasation of the second tracer at permeable sites and so the radiolabelled marker acts to measure blood volume within a test tissue. Calculation of the EVBE value corrects the total tissue ^{125}I-RSA counts for isotope positioned within the vasculature. Therefore, the EVBE represents the extravascularly located ^{125}I-RSA, quantifying BBB permeability.

3.1.1.1 ^{113m}In-Transferrin as a Blood Volume Marker

^{125}I radiolabelled RSA was used as the marker of BBB permeability throughout the double radioisotope experiments and a circulation time of 20-24hrs was employed as in the studies of Leibowitz & Kennedy (1972).The initial blood volume indicator used was ^{113m}In-transferrin which had been proposed as a suitable blood volume marker for use in human studies and also used in multiple tracer *in vivo* experiments (Hosain *et. al.*, 1969; Sisson & Oldendorf, 1971; Oldendorf & Towner, 1974). From the *in vivo* studies utilising ^{113m}In-transferrin, a circulation time of 2mins was chosen (Oldendorf & Towner, 1974).

^{113m}In decays rapidly with a half life of 100mins, necessitating correction for decay during the counting of samples. To assess the rate of decay ^{113m}In standards were placed at regular intervals in the sample count sequence. The standard in position 1 was the 100% reference control to which all other standards were

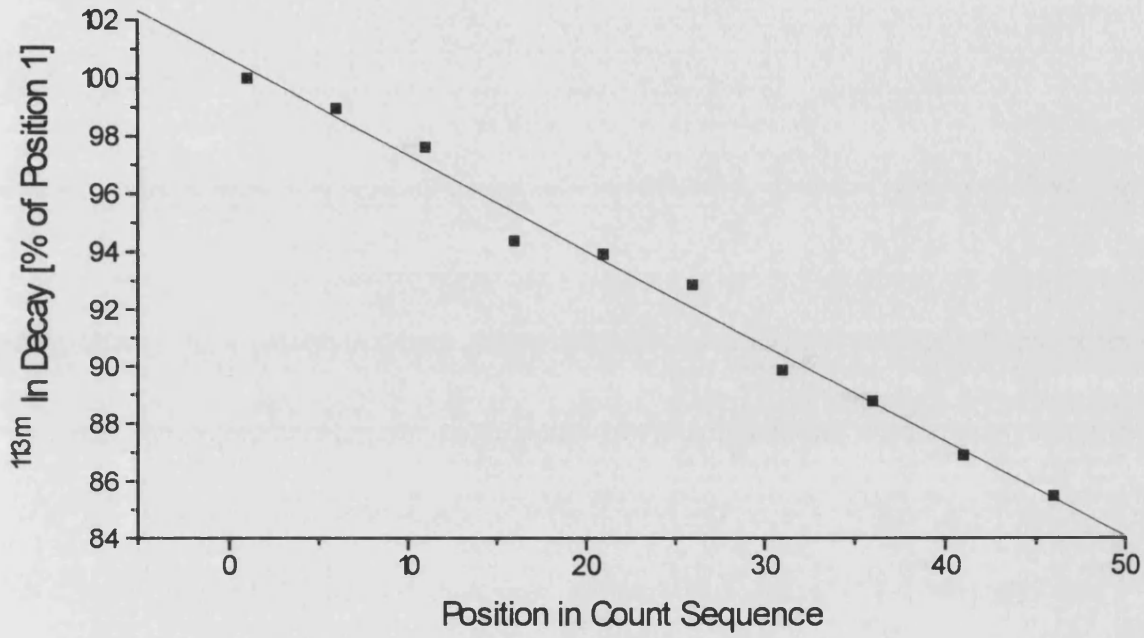


Figure 6: Correction curve for ^{113m}In decay.

^{113m}In standards were placed at positions 1 and 6 in each 10 tube rack. The first standard in the count sequence was referred to as the 100% reference standard. All other standards were calculated as a percentage of the reference and plotted against position in the count sequence. Test samples were corrected according to position in the count sequence. The standard curve shown is taken from a single experiment and is representative of the graphs obtained.

subsequently compared. The data was plotted as a percentage decay curve against position in count sequence and a representative curve is shown in Figure 6.

The primary aim of initial experiments was to repeat the observations of Leibowitz and Kennedy and demonstrate a marked difference in BBB function between normal uninoculated animals and EAE-sensitised rats presenting severe neurological deficits (CHLP). Based on the published data, expectations were of normal EVBE values close to zero and elevated figures for diseased animals.

The results obtained are shown in Figure 7a-c. EVBE values of BBB permeability were negative in all instances indicating over correction by the blood volume tracer. Furthermore, no significant difference was demonstrated between normal and diseased tissues in MP and CSp regions of the CNS. A review of the results omitting correction for the blood content of tissue samples revealed a clear distinction between normal and EAE animals with significance in all areas of the CNS. The results clearly demonstrated that the ^{113m}In tracer correction was adversely distorting the profile of neurovascular opening.

Comparison of ^{125}I BE values from a number of separate experiments (Figure 8), showed little variation between normal groups indicating a high reproducibility of the measurement of albumin within CNS tissue. The reliability of the primary isotope marker plus the detection of differences between normal and EAE groups with uncorrected ^{125}I BE indicated that studies to find a suitable secondary radiolabelled tracer for blood volume correction were justified.

3.1.1.2 ^{111}In-Transferrin as a Blood Volume Marker

Replacement of ^{113m}In with ^{111}In for the labelling of plasma transferrin immediately improved the correction of ^{125}I BE values (Figure 9a-c). The use of ^{111}In-transferrin as the blood volume marker produced consistent EVBE results in normal tissues, demonstrating little variation (Figure 9). Results remained negative although not to the extent seen with ^{113m}In-transferrin (0 to -4 compared with -10 to

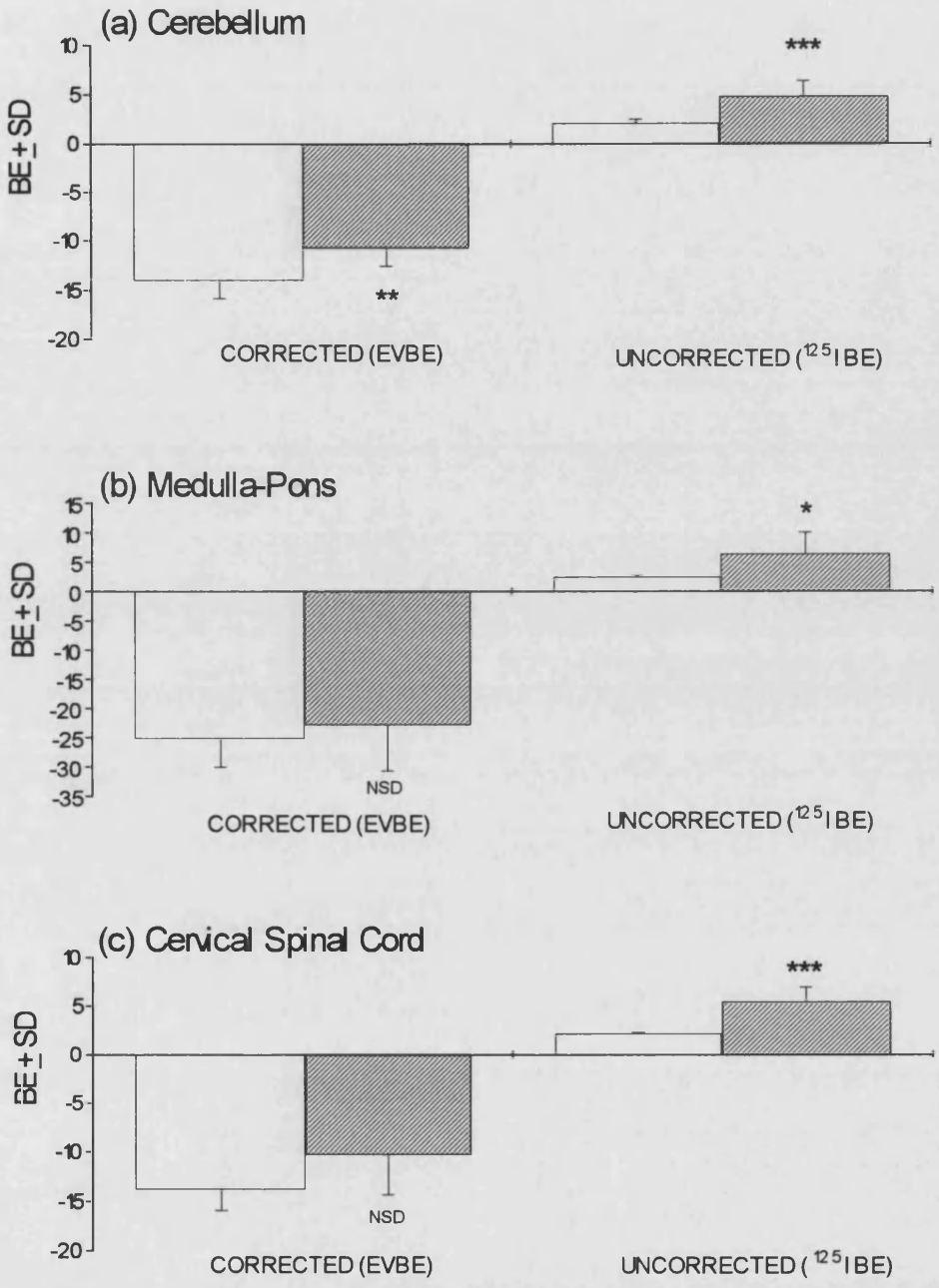


Figure 7: Measurement of BBB permeability using ^{113m}In-transferrin to correct for blood volume. Quantitation of BBB permeability to protein was determined in normal and EAE-sensitised animals at the height of disease (CHLP). ^{125}I-RSA was used as the permeability marker and ^{113m}In-transferrin was employed as the blood volume tracer. Open columns represent normals (n = 8) and hatched columns represent EAE animals (n = 8). The left-hand set of data is corrected for blood volume (EVBE) while the right-hand data is uncorrected (^{125}I BE). Statistical significance was assessed by the Mann-Whitney U test, * $p<0.02$, ** $p<0.01$, *** $p<0.001$, NSD = not significantly different.

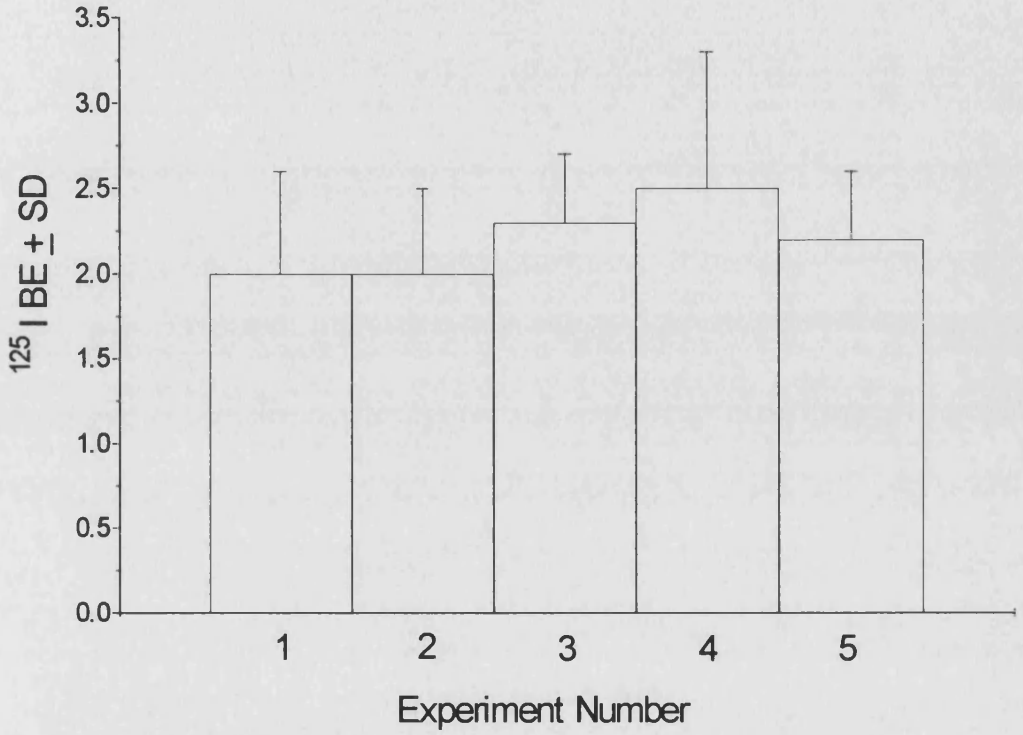


Figure 8: Comparison of Normal ^{125}I BE values Measured in the Cerebellum.

The mean ^{125}I BE values from groups of normal Lewis rats from five separate experiments are shown (n = 3;3;5;6;5 from left to right). Limited variation is demonstrated between experiments with no significant differences being indicated by ANOVA.

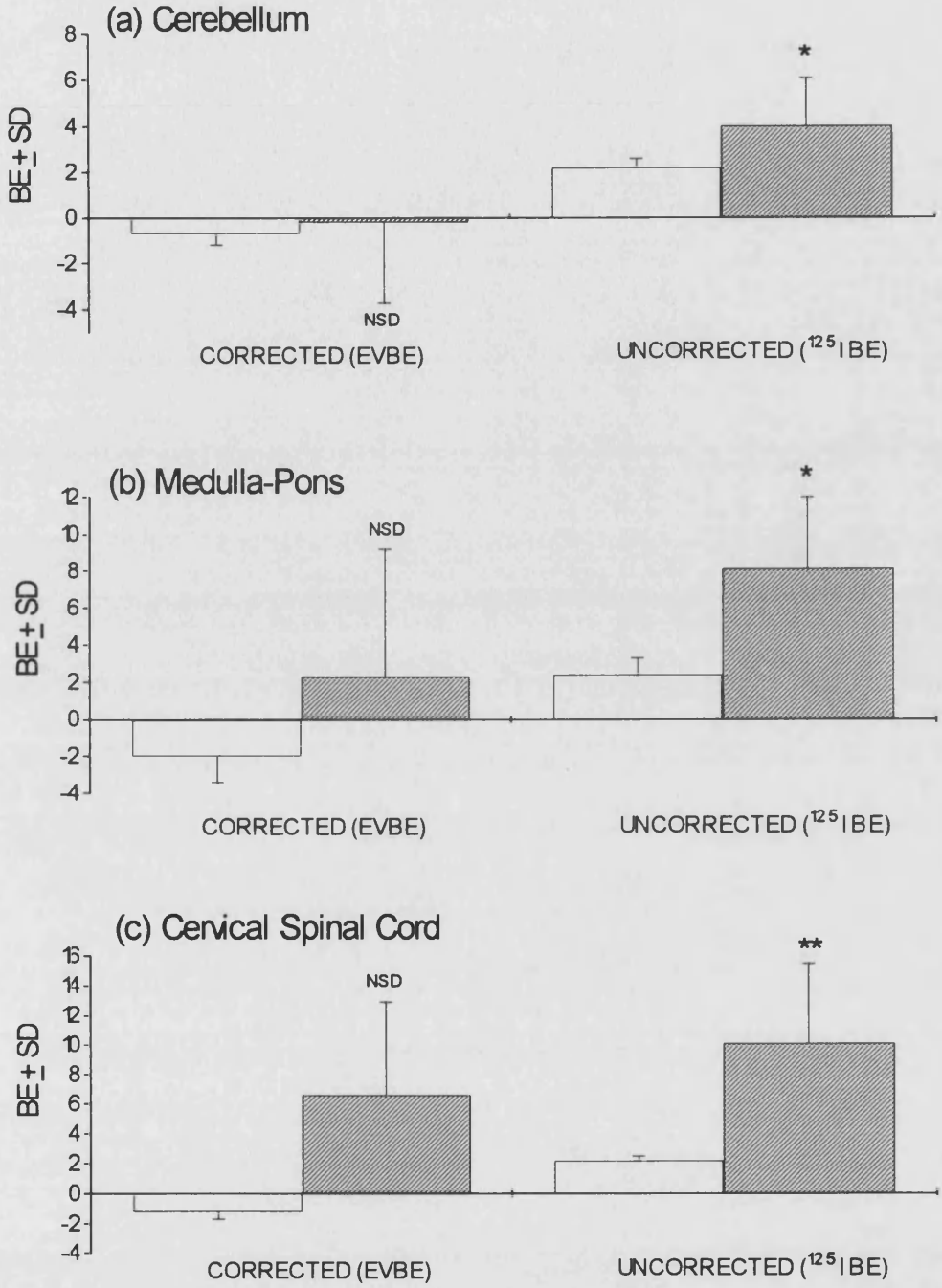


Figure 9a-c: Measurement of BBB permeability using ^{111}In-transferrin to correct for blood volume. Quantitation of BBB permeability to protein was determined in normal and EAE-sensitised animals at the height of disease (CHLP). ^{125}I-RSA was used as the permeability marker and ^{111}In-transferrin was employed as the blood volume tracer. Open columns represent normals (n = 5) and hatched columns represent EAE animals (n = 6). The left-hand set of data is corrected for blood volume (EVBE) while the right-hand data is uncorrected (^{125}I BE). Statistical significance was assessed by the Mann-Whitney U test, * $p<0.02$, ** $p<0.01$, NSD = not significantly different.

-30 previously). Paralysed EAE animals generally expressed positive EVBE values and demonstrated a large variation within the group typical of a disease state. Furthermore, a difference was apparent between normal and EAE in MP and CSp tissues although neither was significant.

^{111}In has a longer half-life than ^{113m}In and could therefore be employed at a lower activity level. Furthermore, no correction of isotopic decay was required for the time period over which the tissue samples were counted (Figure 10).

Analysis of the uncorrected ^{125}I BE values once again indicates clear significant differences between normal and EAE tissues in all CNS areas examined (C and MP $p<0.02$; CSp $p<0.01$).

3.1.1.3 ^{111}In-Transferrin Circulation Times and Isotope Binding Efficiency

A variety of circulation times have been used for the blood volume tracer ranging from 2 - 5mins (Leibowitz, 1969; Sisson & Oldendorf, 1971). Therefore, an experiment was designed to analyse the effect of variations in ^{111}In-transferrin circulation time on the final EVBE results. Groups of normal Lewis rats were used to limit the variation when establishing a profile of BBB permeability for circulation times of 2, 2.5, 3, 5 and 10mins. The results from the 2min circulation, shown in Figure 11, produced EVBE values near to zero in C and CSp tissues but not in the MP (-2.4 ± 0.7). Increasing the duration of the tracer within the vasculature to 2.5 or 3mins did not affect the EVBE except in the CSp where over correction was greater. Circulation times of both 5 and 10mins adversely decreased the EVBE to approximately -3, -5 and -2.5 in C, MP and CSp tissues respectively.

Analysis of ^{111}In binding to plasma protein demonstrated that 55.9% ± 3.2 ($n = 7$) of the label bound with a range of 53 - 63%. Similar determination of binding in blood from animals injected with the ^{111}In-labelled plasma demonstrated no improvement in the percentage of protein bound isotope ($n = 7$: 58.1% ± 5.1, range 53 - 68%).

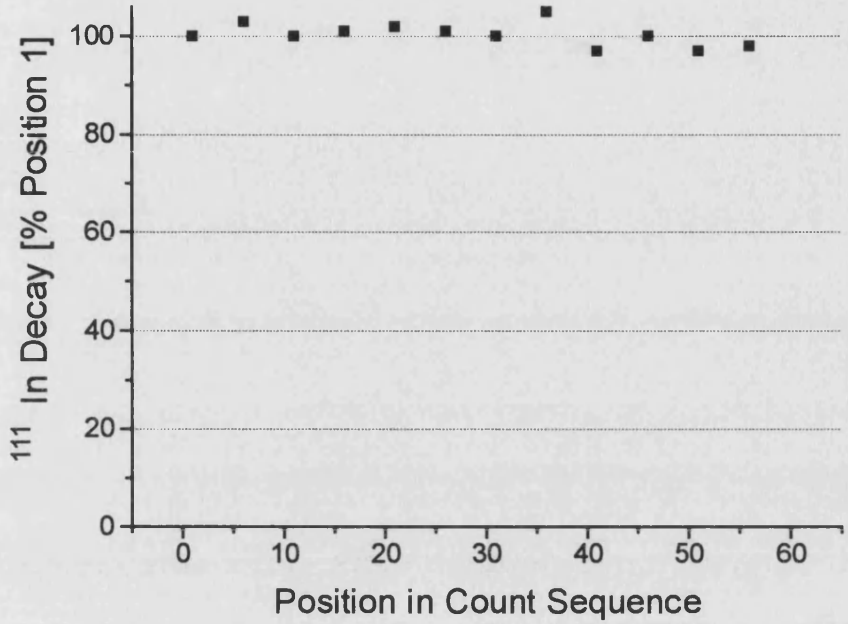


Figure 10: Correction curve for ^{111}In decay.

^{111}In standards were placed at positions 1 and 6 in each 10 tube rack. The first standard in the count sequence was the 100% reference standard. All other standards were calculated as a percentage of the reference and plotted against position in the count sequence.

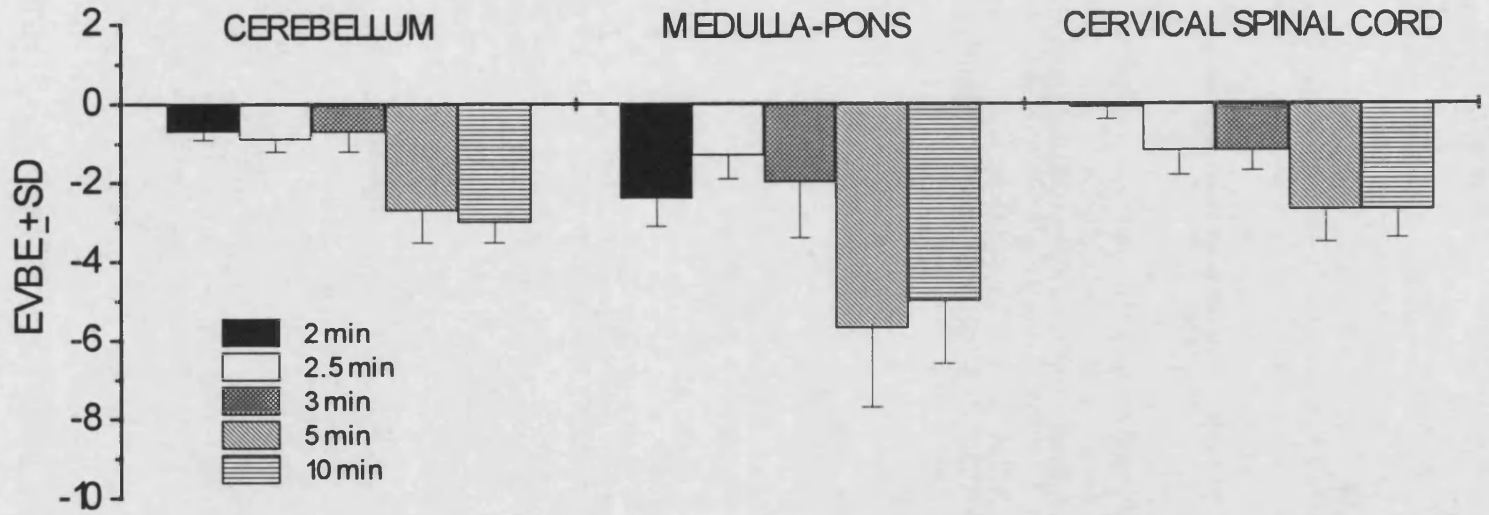


Figure 11: ^{111}In-transferrin Circulation Times

Five groups of normal Lewis rats were used to determine the effect of circulation time of the blood volume marker ^{111}In-transferrin on the measurement of BBB permeability to ^{125}I-RSA. The circulation times selected were 2min (n = 3), 2.5min (n = 3), 3min (n = 5), 5min (n = 6) and 10min (n = 5). ANOVA with a Tukey's pairwise comparison showed 5 and 10min values were significantly increased in all tissues $p<0.05$.

A final study of the suitability of ^{111}In-transferrin as a marker of blood volume was made by assessing the variability between normal and disease groups of ^{111}In BE correction values. Table 2 summarises the results from two representative studies. Clearly a large variation exists between experiments in the range of results calculated, particularly for normal groups. Furthermore, the mean correction values calculated for diseased groups are greater than those of normals in all but one comparison, although significance was not shown. Variability may therefore be linked to neurological status.

Table 2: ^{111}In-transferrin Correction of Blood Volume: Comparison between Normal and EAE-sensitised Lewis Rats

		Mean ^{111}In BE ± SD		
Group	n	C	MP	CSp
Normal[a]	3	3.32 ± 0.95	6.52 ± 3.74	5.20 ± 2.74
EAE D12 PI[a]	6	5.32 ± 4.73	7.71 ± 6.27	4.90 ± 2.87
Normal[b]	6	2.89 ± 0.88	3.90 ± 0.67	2.56 ± 0.68
EAE CHLP[b]	6	4.14 ± 1.86	5.78 ± 3.88	3.60 ± 1.31

[a, b] : Indicates data from same experiment.
NSD was found between normal and EAE values in either experiment; Mann-Whitney U test.

3.1.1.4 ^{111}In-RBC as a Blood Volume Marker

The variable blood volume correction and unsatisfactory level of ^{111}In bound to plasma protein led to an alternative marker being sought. The tracer chosen should not be involved in or affected by the disease process and the amount of labelled tracer

injected tightly controlled. RBC had previously been employed by Rumjanek *et. al.* (1984a) in a similar study and the cells met the listed criteria. Furthermore, methodology had been published describing the labelling of RBC with ^{111}In-tropolonate (Osman & Danpure, 1987). The circulation time for ^{111}In-RBC was raised to 5min according to the original methodology of Leibowitz and the study by Rumjanek employing radiolabelled RBC for blood volume determination (Leibowitz & Kennedy, 1972; Rumjanek *et. al.*, 1984).

The corrected EVBE results achieved utilising the RBC marker demonstrated a marked improvement over previous methods. Figure 12 shows significant differences in BBB permeability between normal rats and EAE-sensitised animals at the height of disease (neurological status range 2 - 4) in all CNS areas examined ($p<0.05$). Indeed, the profile presented by the uncorrected ^{125}I BE results remains after correction. Normal EVBE values are close to zero and variability within tissue groups is minimal (C = 0.8 ± 0.1; MP = 1.0 ± 0.3; CSp = 1.3 ± 0.4; n = 3). In contrast diseased animals demonstrate a high EVBE indicating enhanced neurovascular permeability to systemic albumin. The variability within the EAE group is increased due to the differing responses of individual rats to disease development (C = 4.0 ± 2.0; MP = 11.4 ± 6.9; CSp = 11.8 ± 4.9; n = 7). Interestingly, the extent of BBB opening is greater in both MP and CSp tissues than in the C.

Finally, Table 3 shows that no significant difference exists in ^{111}In-RBC correction values for blood volume (^{111}In BE) between normal and EAE-inoculated groups at D12 PI in any CNS tissue analysed.

Furthermore, the method of RBC labelling ensures that the ^{111}In endproduct is 100% bound to cells, the unbound isotope having been washed out. Figure 13 represents the typical level of activity achieved per 5 x 10^9 cell preparation. Daily, but minimal variations in activity exist between preparations of ^{111}In-RBC. However, the final activity was never less than 5μCi per injection.

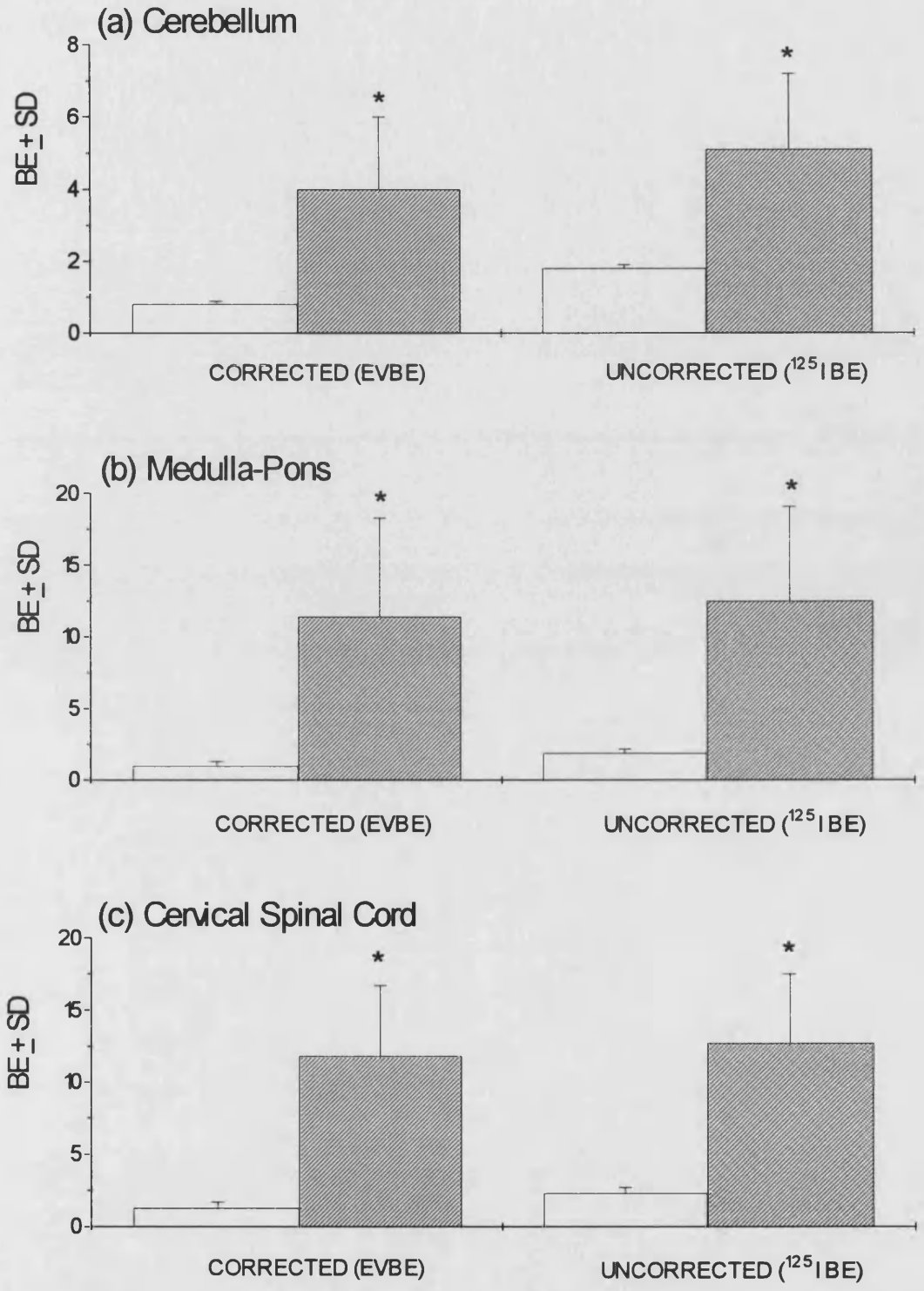


Figure 12a-c: Measurement of BBB permeability using ^{111}In-RBC to correct for blood volume. Quantitation of BBB permeability to protein was determined in normal and EAE-sensitised animals at the height of disease (HLW - CHLP). ^{125}I-RSA was used as the permeability marker and ^{111}In-RBC were employed as the blood volume tracer. Open columns represent normals (n = 3) and hatched columns represent EAE animals (n = 7). The left-hand set of data is corrected for blood volume (EVBE) while the right-hand data is uncorrected (^{125}I BE). Statistical significance was assessed by the Mann-Whitney U test, * $p<0.03$.

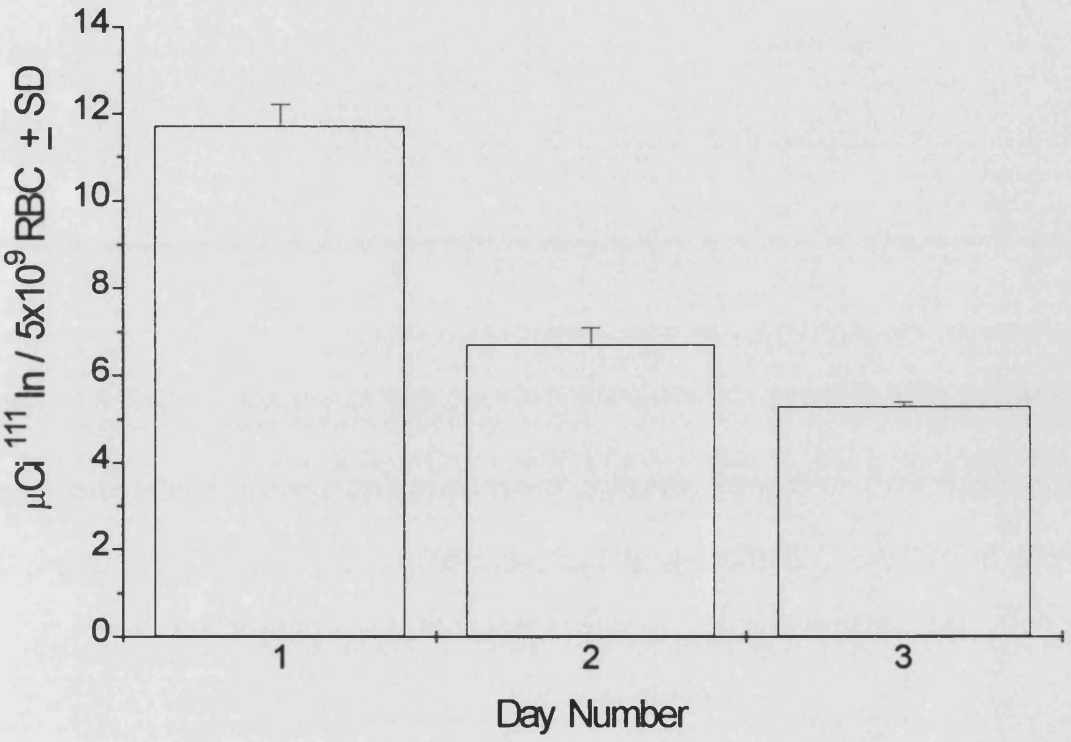


Figure 13: Final Activity of ^{111}In / 5 x 10^9 RBC Preparations

Labelling of RBC on day 1 employed a fresh batch of ^{111}In. Radiolabelling on subsequent days used the same batch which had accumulated decay product with time. All groups (n = 5) are representative of the final activity achieved in all experiments conducted and a single batch of ^{111}In was typically only used for three consecutive days.

Table 3: ^{111}In-RBC Correction of Blood Volume - Comparison between Normal and EAE-sensitised Lewis Rats

		Mean ^{111}In BE ± SD		
Group	n	C	M	CSp
Normal[a]	6	0.98 ± 0.16	0.87 ± 0.13	0.97 ± 0.13
EAE D12 PI[a]	14	1.08 ± 0.24	1.15 ± 0.33	0.81 ± 0.24
Normal[b]	5	0.87 ± 0.39	0.62 ± 0.51	0.63 ± 0.25
EAE D12 PI[b]	10	0.78 ± 0.18	0.67 ± 0.12	0.71 ± 0.13

[a, b] : Indicate data from separate experiments
NSD was found between normal and EAE values in either experiment; Mann-Whitney U test.

The final method developed employing ^{125}I-RSA as the marker of extravasation and ^{111}In-RBC as the blood volume tracer was able to quantitate protein accumulation in a range of CNS tissues. The technique is highly reproducibility and can demonstrate large differences in BBB permeability between normal and EAE-paralysed rats.

3.1.2 Water Extravasation

An alternative measure of BBB breakdown in the CNS during EAE is the quantitation of edematous water influx into CNS tissues. Such an approach could either be employed alone to provide a reliable method for examining pharmacological restriction of edema build-up, or be used to support the findings of ^{125}I-RSA permeability studies. Three different techniques were employed to measure the edematous aspect of BBB dysfunction.

3.1.2.1 Dual-tracer Analysis of Edema Formation

To determine extravascular accumulation of $^{3}H_2O$ together with ^{125}I-RSA in tissues from a single animal, a dual count parameter was constructed on an LKB Rackbeta 1219. A standardisation for quench error was performed for both ^{3}H and ^{125}I when designing the parameter settings (Figure14a,b). Analysis of combined ^{125}I and ^{3}H standards on the dual count parameter demonstrated an accurate recognition of ^{125}I levels and a consistent yet slightly dampened dpm for ^{3}H (Figure 14c). A reduced ^{3}H value would not affect subsequent calculation of the BE ratio if the dampening was consistent as indicated.

Application of the dual count method to the quantification of BBB permeability during EAE did not provide a profile of water extravasation (Figure15). The results obtained by the dual-tracer method highlight no significant difference between normal animals and experimental groups in any CNS area studied. Furthermore, the normal results appear generally higher than those of animals presenting neurological deficits.

The technique also produced unacceptable levels of chemiluminescence and colour quench in many samples. Furthermore, the possibility that ^{125}I levels within samples may also have affected $^{3}H_2O$ measurement cannot be ruled out. However, ^{125}I-RSA activity could not be reduced as the detection of radiolabelled protein would have been affected. The lack of a clear difference between normal and disease states deterred further investigation using this technique.

3.1.2.2 Ethanol Extraction of $^{3}H_2O$ for Edema Measurement

A modification of the method used by Simmons *et. al.* (1982), avoided the chemiluminescence and colour quench problems encountered by the dual count method. A single isotope, $^{3}H_2O$, was injected into the circulation and the extravascularly accumulated water extracted from dissected CNS areas by dehydration with ethanol. The ^{3}H content of ethanol extracts and plasma aliquots was subsequently assessed on a single count parameter.

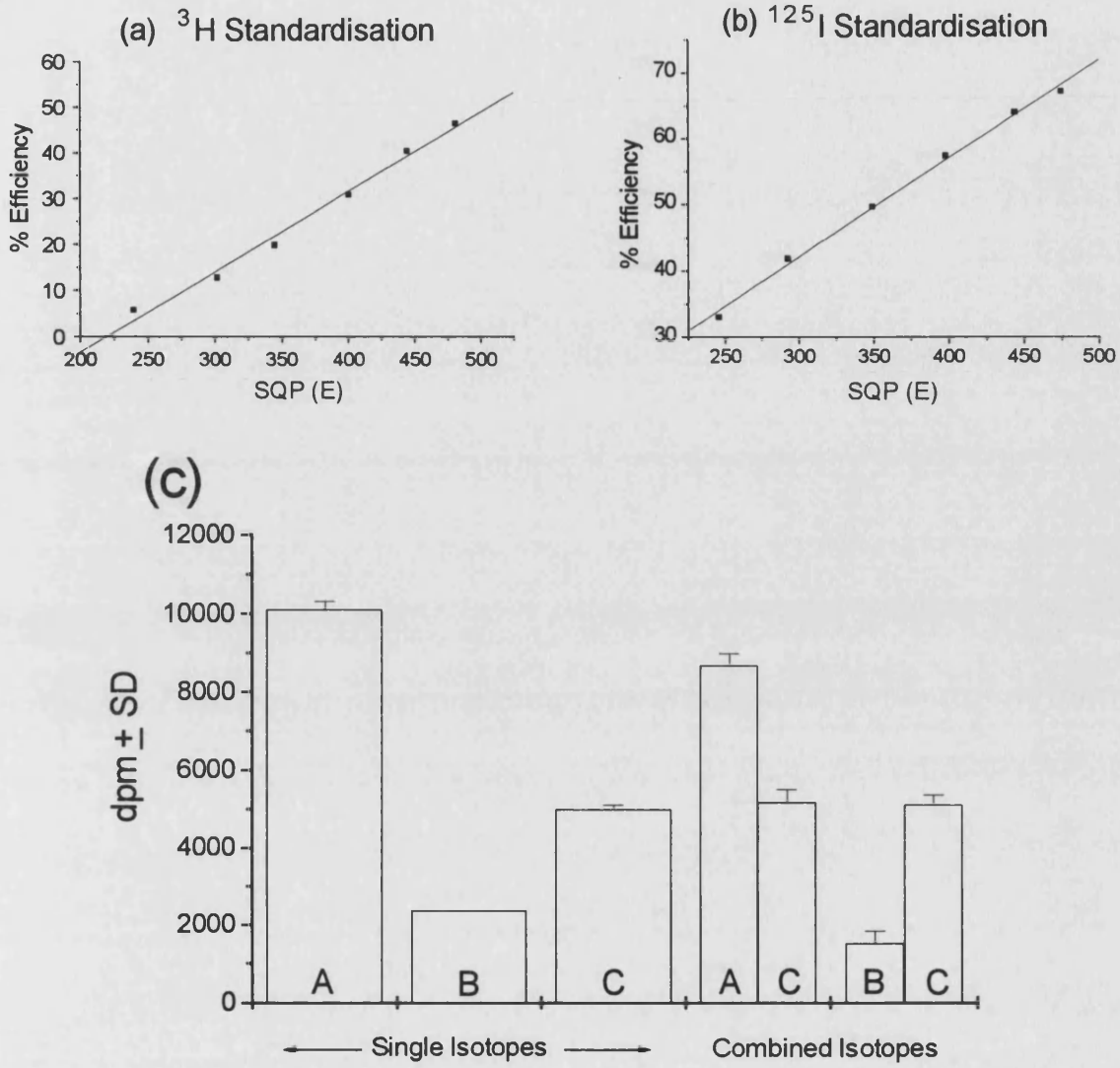


Figure 14: Standardisation of Dual Count Parameter for the Measurement of ^{3}H and ^{125}I Radioisotopes. The two line graphs **(a)** and **(b)** show the standardisation of spectral quench parameters (external) (SQP(E)) for ^{3}H and ^{125}I respectively, using a dual count parameter on an LKB Rackbeta 1219. Carbontetrachloride was used as the quenching agent in a range from 0 to 200µl per 10ml of scintillant. The histogram **(c)** shows the ability of the dual count parameter to detect both ^{3}H and ^{125}I within a mixed sample. Three standards were employed: A = 10 000dpm ^{3}H; B = 2 500dpm ^{3}H; and C = 5 000dpm ^{125}I.

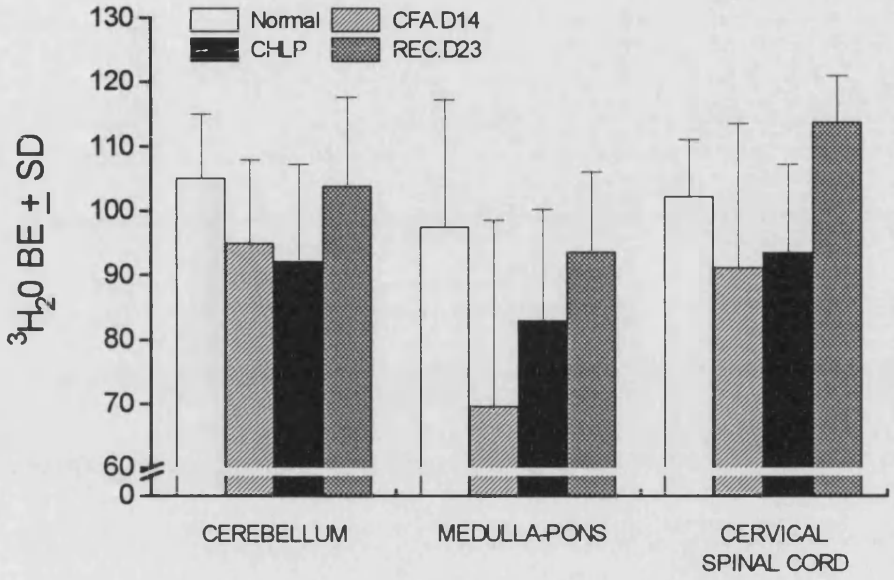
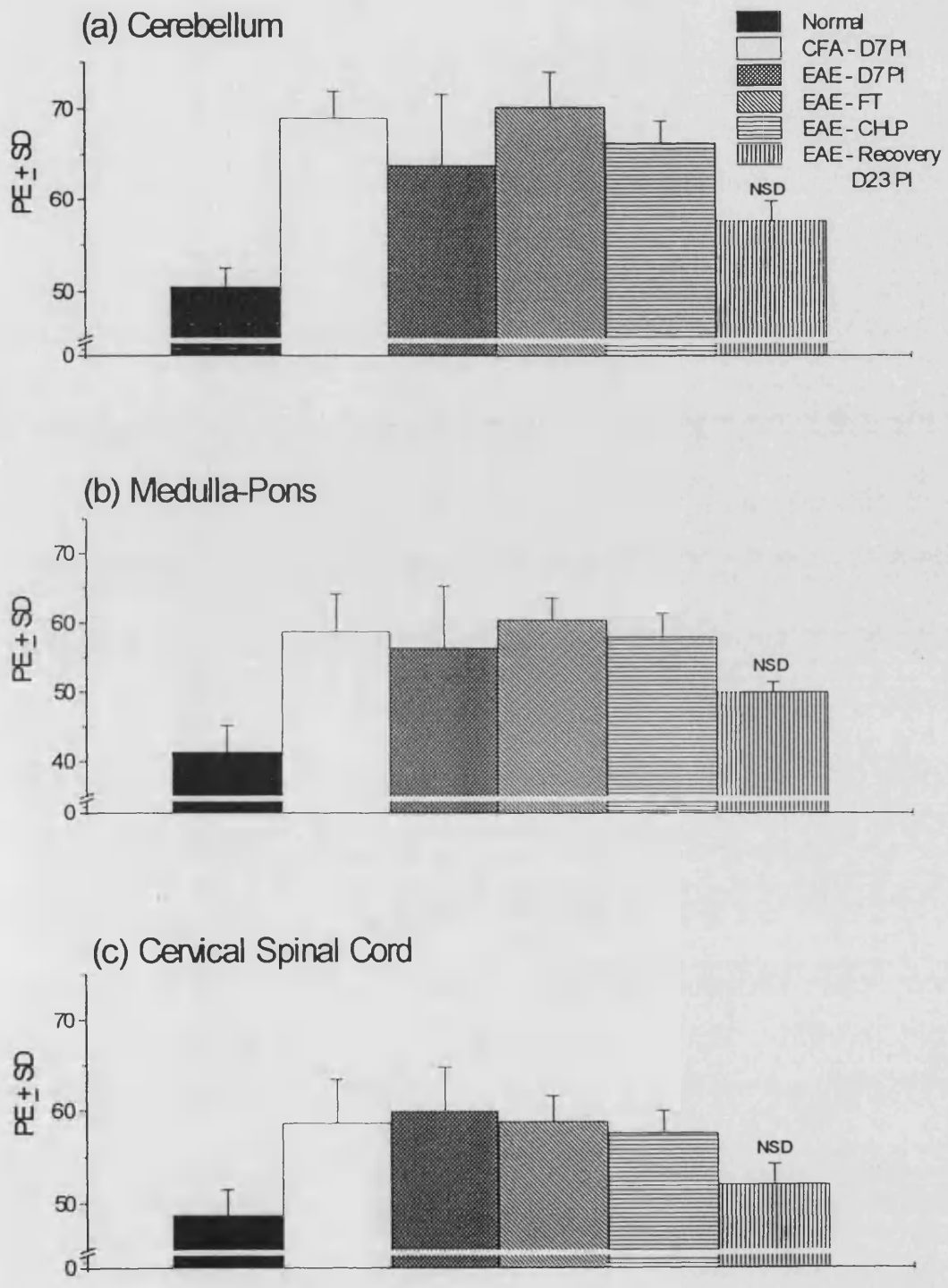


Figure 15: Water Extravasation During EAE Measured by a Dual-tracer Technique. Water accumulation within CNS tissues of EAE, CFA control and normal groups was measured by liquid scintillation counting of ^3H_2O in solubilised samples, together with a determination of ^{125}I-RSA extravasation (data not shown). Normal (open column, n = 9), CFA-inoculated (diagonally hatched column, n = 6), paralysed EAE-inoculated (black column, n = 8) and recovered (REC) EAE-inoculated D23 PI (cross hatched column, n = 6) animals were analysed. The data was subjected to an ANOVA resulting in no significant differences between experimental groups and normal values being shown.

The first experiment assessed EAE-sensitised animals at a range of timepoints with comparison to normal and CFA-inoculated controls. Five CNS areas were examined producing a comprehensive profile of water extravasation (Figure16a-e). Normal tissues showed low values in comparison to disease figures, with little variation within the tissue data groups of normal animals. The profile showed that brain water accumulation increased as early as D7 PI in EAE-inoculated animals. However, an increase in ^3H_2O PE in CFA-inoculated controls at D7 PI was also noted, indicating non-specific adjuvant effects. The edema content of CNS tissues remained elevated throughout early (FT) and severe (CHLP) stages of neurological disease ($p<0.05$, compared to normal). The CNS water content decreased with loss of neurological deficits and was not found to be significantly different from normal values at D23 PI, during early recovery.

Confirmation of the initial findings could not be demonstrated in subsequent experiments, questioning the reproducibility of the technique. Figure 17 illustrates the variability of normal control values obtained from five representative experiments. Variation within individual groups is limited, but between experiments the differences are significant ($p<0.05$).

The technique not only produced discontinuity between experiments but also within studies. Figure 18a-b combines the results of three separate investigations using ethanol extraction to quantify edema formation. The differences highlighted between normal rats and animals with CHLP in the original profile - (shown as study 1 in Figure 18) - was not observed in later studies - (2 and 3, Figure 18). The differences in experiments 2 and 3, were either reduced to a non-significant level or abolished in both the C and TSp tissues. The technique lacked the reproducibility required for pharmacological studies and a third approach to edema measurement was assessed.


(a) Cerebellum
Normal
CFA - D7 PI
EAE - D7 PI
EAE - FT
EAE - CHLP
EAE - Recovery D23 PI
NSD
PE +- SD
70
60
50
0
(b) Medulla-Pons
NSD
PE +- SD
70
60
50
40
0
(c) Cervical Spinal Cord
NSD
PE +- SD
70
60
50
0

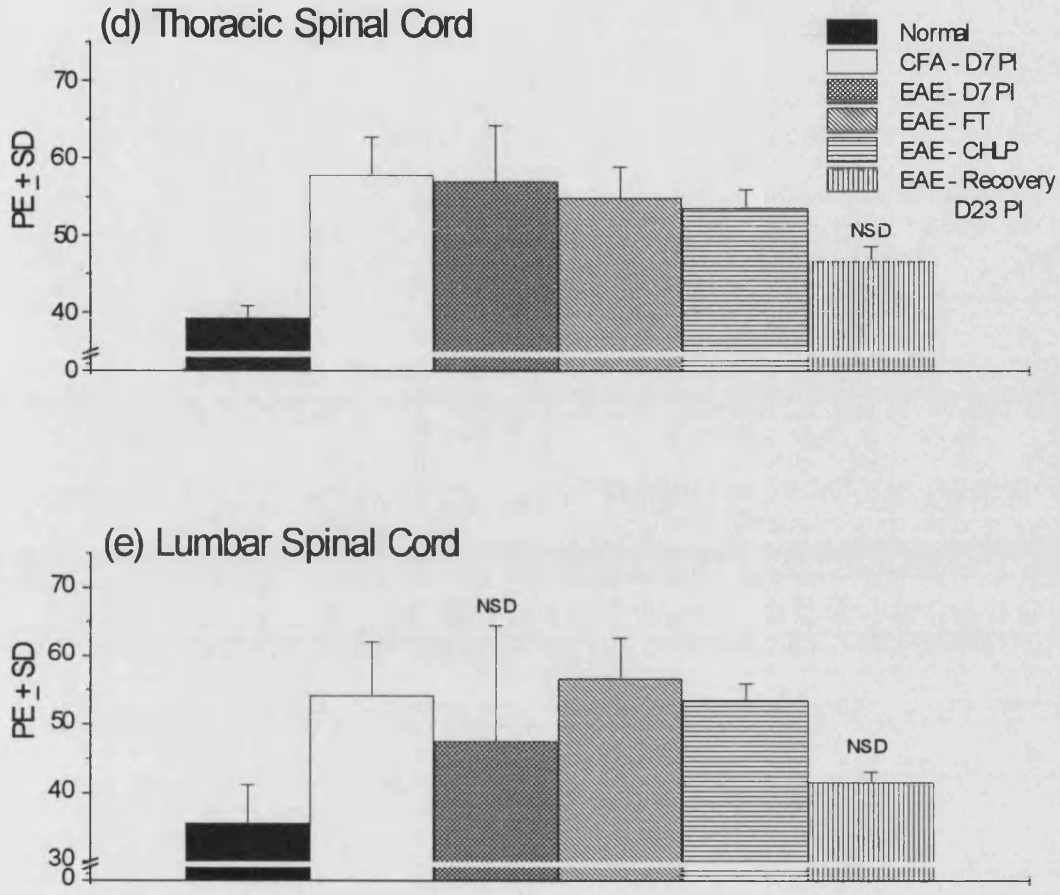


Figure 16: Water Extravasation During EAE Measured by Ethanol Extraction Technique. Accumulated ^3H_2O was removed from CNS tissues **(a)** cerebellum, **(b)** medulla-pons, **(c)** cervical spinal cord, **(d)** thoracic spinal cord and **(e)** lumbar spinal cord, by ethanol extraction and the results expressed as plasma equivalents (PE). Six groups of Lewis rats were examined: normals (n = 5); CFA-inoculated D7 PI (n = 4); EAE-inoculated D7 PI (n = 6); EAE- FT (n = 7); EAE-CHLP (n = 8); and EAE- recovery D23 PI (n = 3). All groups were significantly different from normals in all tissues examined ($p<0.05$) with the exception of recovery groups in all areas and EAE D7 PI in the lumbar spinal tissue (NSD); ANOVA followed by Dunnett's multiple comparison to a single control.

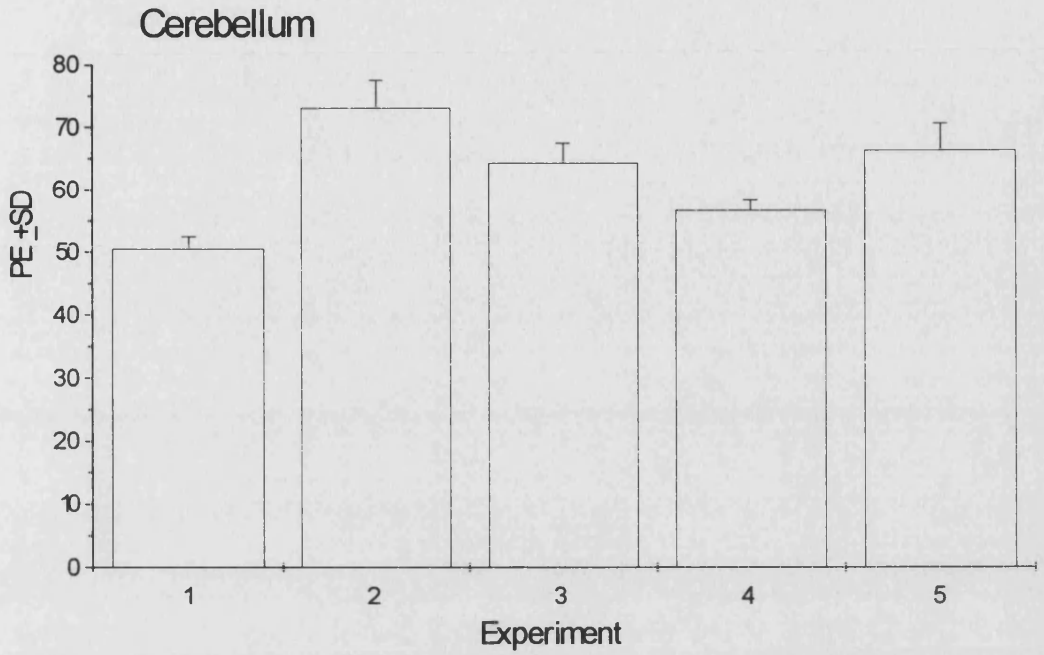


Figure 17: Variability of Normal ^3H_2O Values in the Cerebellum following Ethanol Extraction. Estimation of water extravasation by ethanol extraction of accumulated ^3H_2O in the cerebellum of normals from five separate experiments (n = 5, 3, 3, 6 and 6 left to right). Differences were shown to exist by ANOVA at $p<0.05$. The cerebella results shown are typical of the variation displayed by all tissues between experiments.

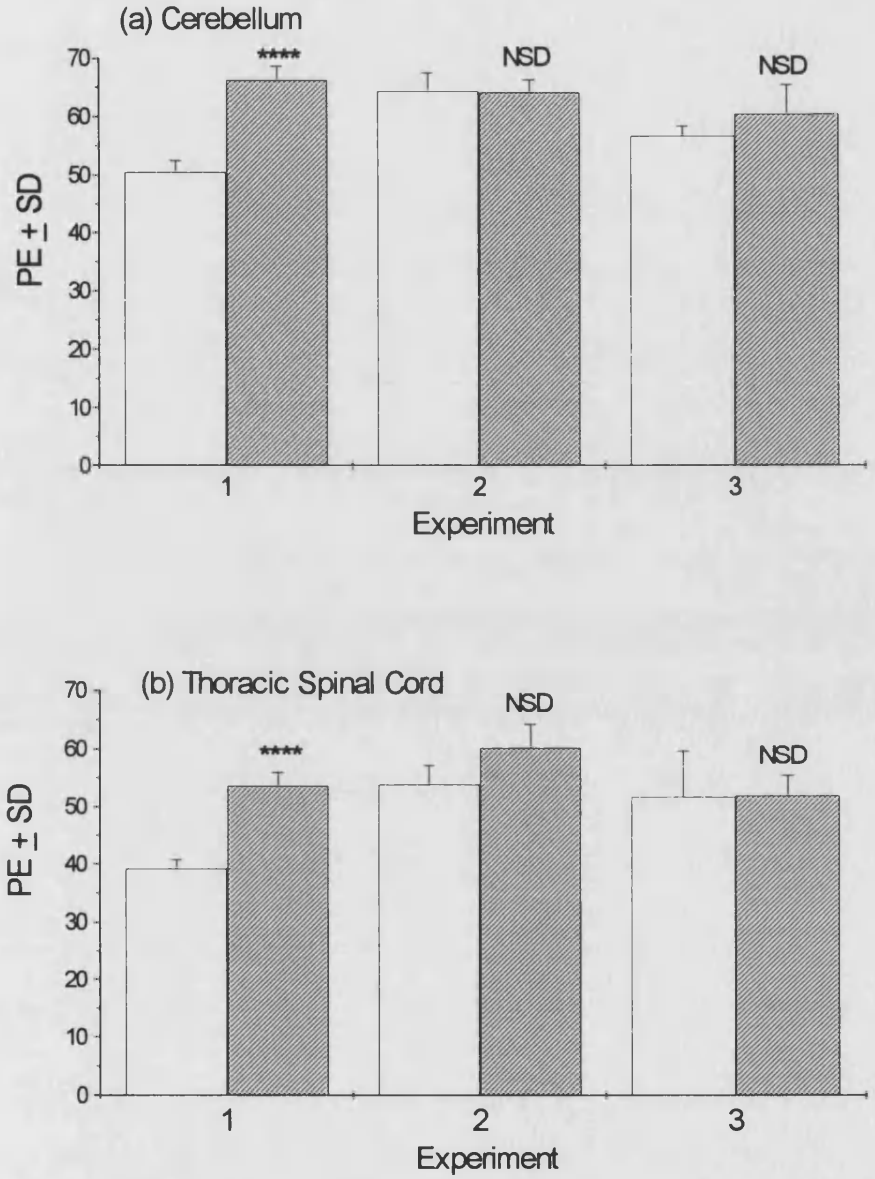


Figure 18: Variability within Experiments when comparing Normal and EAE Values of Water Extravasation by the Ethanol Extraction Technique.
The results of three studies comparing normal Lewis rats (open column) with EAE-animals showing neurological deficits (hatched column) are illustrated for the **(a)** cerebellum and **(b)** thoracic spinal cord tissues. Study 1 represents the results from the initial profile reported in Figure 16a and d; normal (n = 5), EAE (n = 8). Studies 2 and 3 aimed to repeat the findings of study 1; normal (n = 3, 6), EAE (n = 6, 3) respectively. Highly significantly differences were found in study 1 (**** p = 0), but no significant difference (NSD) was observed between normal and EAE groups in either study 2 or 3; T-Test.

3.1.2.3 % Water Content

Levine had previously reported edema formation within the CNS by calculating % water content from tissue wet and dry weights (Levine *et. al.* 1966, 1977). Assessment of CNS tissue water content in EAE-sensitised animals by weight was made at timepoints D7PI and D12PI which had both been implicated in water accumulation in the CNS by the ethanol extraction technique. At D7 PI no difference in the water content of C or CSp tissues from normal and EAE animals was noted (Figure 19a,b), a result typical of other CNS areas (Table 4). Moreover, the trend in the CSp was for a lower % water content in the disease group compared to the normals.

Table 4: Assessment of CNS % Water Content in Normal and EAE-inoculated Lewis Rats at D7 and D12 PI

		%Water Content ± SD		
Group	n	MP	TSp	LSp
Normal[a]	3	73.4 ± 1.5	71.4 ± 1.38	73.2 ± 1.22
EAE D7PI[a]	7	73.4 ± 0.4	70.6 ± 0.8	73.5 ± 1.6
Normal[b]	3	73.0 ± 0.5	70.1 ± 1.7	73.4 ± 0.8
EAE D12PI[b]	7	73.7 ± 1.5	73.9 ± 0.7	76.1 ± 1.0*
Normal[c]	6	75.6 ± 0.5	71.8 ± 0.7	75.8 ± 1.7
EAE D7PI[c]	5	75.5 ± 1.9	72.0 ± 1.8	77.3 ± 2.9
EAE D12PI[c]	3	75.2 ± 1.4	73.3 ± 0.9	77.2 ± 1.8

[a-c] : Indicates data from same experiment.
No significant differences between normal and EAE groups were found except between normal and EAE D12 PI in the lumbar spinal cord in one out of two replicate studies (* $p<0.05$); T-test.

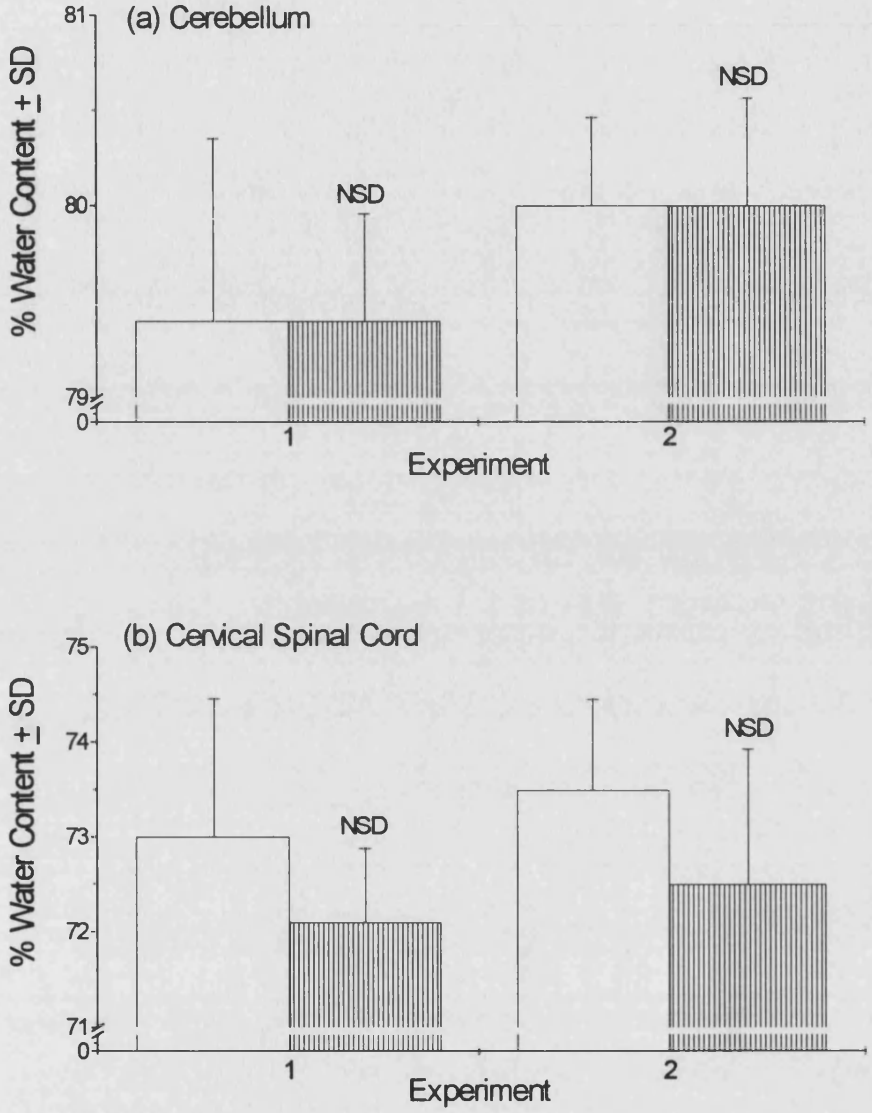


Figure 19: Water Extravasation During EAE Assessed by % Water Content - Comparison of Normal and EAE at D7 PI.

From wet and dry weight measurements the % water content of CNS tissues **(a)** cerebellum and **(b)** cervical spinal cord was assessed in normal (open column) and EAE at D7 PI (Vertical line column) animals. The results of two independent experiments are shown. Study 1: normal, n = 3; EAE D7 PI, n = 7. Study 2: normal, n = 6; EAE D7 PI,
n = 5. NSD - no significant difference was found between normal and EAE groups, T-test.

The results of the comparison between normal and early neurological disease in EAE were also inconclusive (Figure 20a,b). Values in the C indicated a lower % water content in diseased animals (78.2% ± 0.6; 79.2% ± 0.55) compared to normals (79.1% ± 0.8; 80% ± 0.5). A significant increase in the water content of EAE tissue at D12 PI was noted in the LSp ($p<0.05$), but could not be consistently shown (Table 4). In conclusion, analysis of % water content as a measure of edema accumulation was unsatisfactory showing no reliable indication of a significant difference between normal and disease states in any tissue.

In summary, the measurement of water influx into the CNS parenchyma as a result of EAE development has been analysed by three separate techniques. All methods lacked significant reproducibility and a difference between normal and EAE states were not necessarily shown. However, the procedure developed from published methodology to quantify protein extravasation across the BBB is reliable and consistently demonstrates a large difference in permeability between normal and disease states. Therefore, measurement of albumin movement across the BBB is the method employed in the following chapters.

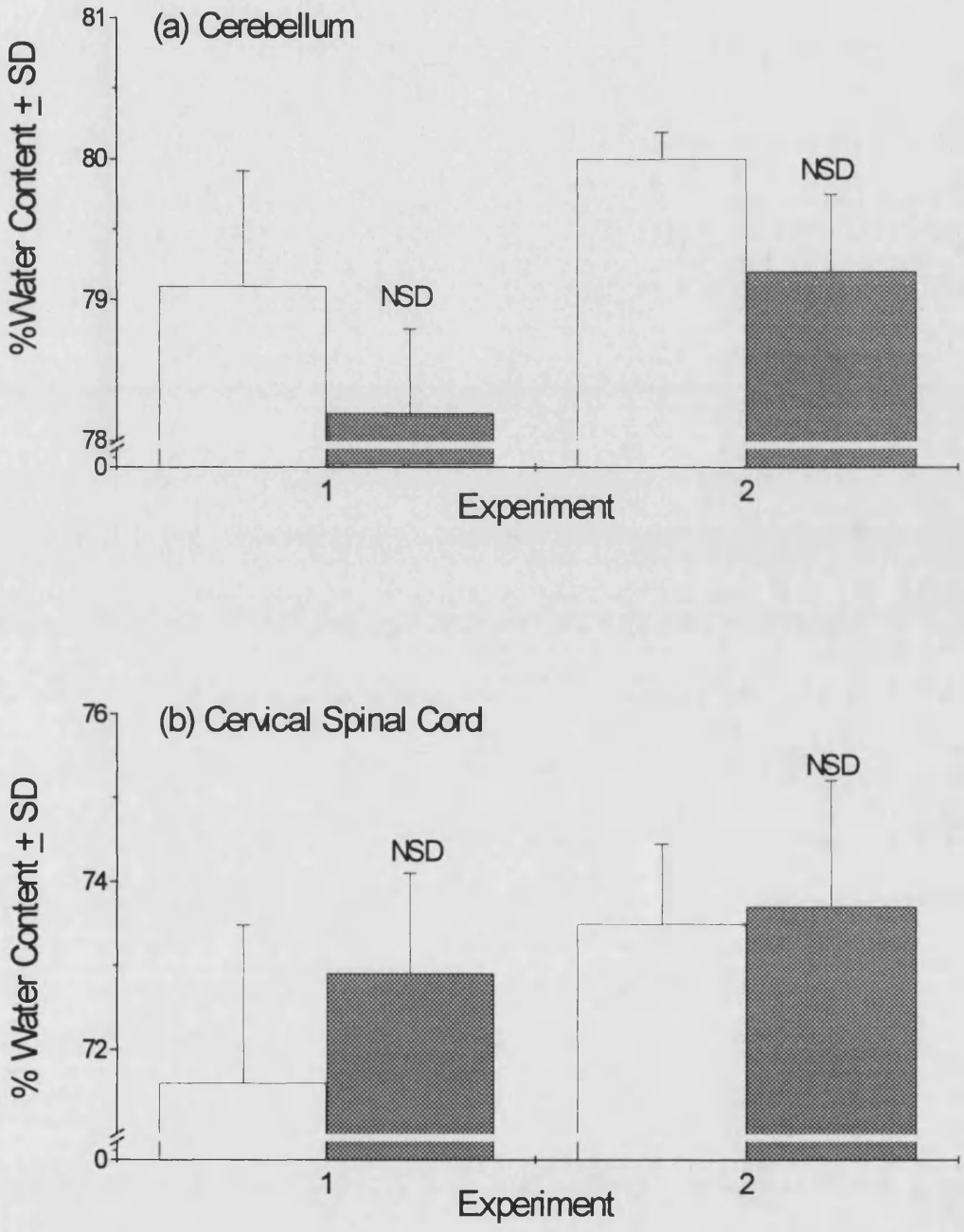


Figure 20: Water Extravasation During EAE Assessed by % Water Content - Comparison of Normal and EAE at D12 PI.

From wet and dry weight measurements the % water content of CNS tissues **(a)** cerebellum and **(b)** cervical spinal cord was assessed in normal animals (open column) and EAE-sensitised rats D12 PI (cross hatch column). The results of two independent experiments are shown. Study 1: normal, n = 3; EAE D12 PI, n = 7. Study 2: normal, n = 6; EAE D12 PI, n = 3. NSD - no significant difference was found between normal and EAE groups, T-test.

3.2 Discussion

The T cell mediated neuropathology of EAE presents many potential sites for assessing the progress of disease and the performance of pharmacological agents. Abnormal BBB function is consistently a key development in the progression of EAE and therefore an ideal parameter to study and control.

As a result of BBB perturbation vascularly-derived water, proteins and immune cells influx into the inflammatory EAE lesion. Measurement of the accumulation of any systemically-derived inflammatory components in the CNS parenchyma could be used to indicate disease progression. Analysis of water and albumin build-up in extravascular sites was chosen as, unlike cellular infiltrates, the pharmacological findings pertaining to these components may be applicable to other conditions involving impaired neurovasculature, including head injury and meningitis.

The ideal experimental design would have assessed both water and albumin movement across the BBB within the same CNS samples. However, the scintillation counting of radioisotopes ^{125}I and ^{3}H contained in whole blood and CNS tissue samples, presented a range of variables and errors, amongst which the greatest problems were chemiluminescence and colour quench. Chemiluminescence is the conversion of chemical energy to molecular electronic excitation energy which creates a photon of light. Consequently, the additional light interferes with the accurate detection of photon emissions derived from nuclear particle kinetic energy. ^{3}H determinations are particularly affected. The high levels of chemiluminescence associated with the dual radioisotope technique were attributed to the chemical solubilizer used and the high lipid content of the CNS samples. Colour quench of the scintillant-signal was an inherent feature of the samples under examination. Bleaching of samples with hydrogen peroxide was considered, but further aggravation of the chemiluminescence by chemical addition was determined to be inappropriate.

The lack of distinction between normal and diseased animals by the dual scintillation measurement of both albumin and water, indicated that the protocol was not suited to the tissues under assessment. Lengthy chemical manipulations to reduce the complicating quench and chemiluminescence errors were therefore not investigated.

A single isotope method adapted from work by Simmons *et. al.* (1982) enabled measurement of water extravasation in CNS tissues without the complications encountered in the dual radioisotope procedure. Extraction of tritiated water from tissue with ethanol and the calculation of plasma rather than whole blood equivalents omitted colour quench of the scintillation counting. The results from the Simmons study in acute EAE distinguished a small definition between normal and disease states at the distal end of the spinal cord (Simmons *et. al.* 1982). The adapted method presented here recognised edema formation in all areas of the CNS examined. While subsequent analysis using the procedure demonstrated an unacceptable variability, a number of aspects relating to the original profile were found to be in agreement with published observations.

The current study found elevated neurovascular permeability for water at D7 PI prior to the onset of EAE symptoms, which confirmed previous work using Gd-DTPA and mannitol (MW 550 and 182Da respectively) (Daniel *et. al.*, 1981; Lam, 1986; Hawkins *et. al.*, 1991). Furthermore, Gd-DTPA leakage returned to normal levels by recovery (D20PI), which agrees with the present observations (Hawkins *et. al.* 1991).

Increased CNS tissue water content in samples from CFA-inoculated animals D7PI is in agreement with a report by Suckling *et. al.* (1984), who measured elevated CSF albumin concentrations in CFA controls at an identical timepoint. CFA-induced changes are not regularly reported for large proteins at pre-disease timepoints (Oldendorf & Towner, 1974; Traugott, 1989). However, the water extravasation seen

at D7PI in CFA controls may implicate adjuvant-induced perturbation of barrier function during the initiation of EAE.

The majority of small tracer studies in EAE report significant changes in BBB permeability at the distal end of the spinal cord at D7PI and only limited involvement of other CNS regions during an acute attack (Oldendorf & Towner, 1974; Daniel *et. al.*, 1981; Simmons *et. al.*, 1982). However, the edema measurements reported here express a general fluid accumulation in all CNS areas. The disparity with published findings may be indicative of a methodological anomaly rather than a greater sensitivity, which when considered with the inconsistency of results, renders the technique unsuitable for assessment of drug actions at the BBB.

Calculation of tissue water content by wet and dry weight measurements has been successfully implemented by investigators assessing CNS edema formation following induction of hyperacute EAE (Levine *et. al.*, 1966), chemical irritation (Levine & Torrelio, 1977) and compression head injury (Yamaguchi *et. al.*, 1976). Published values in the range 78-79% for normal brain water content are in close agreement with the normal levels reported in the present study (Levine & Torrelio, 1977; Yamaguchi *et. al.*, 1976). Edematous CNS tissues typically demonstrate a 1-4% increase in water content above normal values when calculated by this method (Levine *et. al.*, 1966; Yamaguchi *et. al.*, 1976; Levine & Torrelio, 1977). The current study in the Lewis rat model of acute EAE demonstrated no distinction between normal and diseased tissues. The size of tissue analysed was notably smaller than the areas studied by Levine *et. al.* (1966) and Yamaguchi *et. al.* (1976). Furthermore, the proteinaceous plasma exudate and inflammatory cells entering an EAE perivascular lesion area may significantly contribute to the dry weight measurements, obscuring the increase in tissue water. Indeed, a number of reports detect a measurable increase in tissue DNA as a result of inflammatory cell infiltration (Oldendorf & Towner, 1974; Smith *et. al.*, 1984; Honegger *et. al.*, 1989b).

Determination of neurovascular dysfunction by recording accumulated levels of ^{125}I-RSA successfully and reliably distinguished between normal and acute EAE states, agreeing with the single isotope measurements made by Leibowitz & Kennedy (1972). However, a number of trials were necessary to identify a suitable second tracer for the correction of tissue blood volume.

Indium labelling of plasma transferrin had been successfully employed by Hosain *et. al.* (1969), Sisson & Oldendorf (1971) and Oldendorf & Towner (1974) to measure blood distribution spaces. However, in this study following a 1:1 (v/v) binding procedure only 50-60% of the ^{111}In label was protein-bound, indicating a saturation of binding to plasma protein, which has been reported to be transferrin (Adatepe *et. al.* ,1969). Therefore, a significant amount of the isotope was either unbound or attached to a non-protein plasma constituent, potentially increasing the access of the label to the extravascular compartment of the CNS. Indeed, the concentration of ^{113m}In employed was ten-fold greater than the amount of ^{111}In used to compensate for the rate of decay. Consequently, the binding of ^{113m}In to protein might be expected to be even lower than that achieved with ^{111}In. A difference in protein labelling would explain the improvement in blood volume correction observed when ^{111}In replaced ^{113m}In as the blood volume tracer radiolabel.

Oldendorf & Towner (1974), did not report limited binding of ^{113m}In to plasma transferrin when employing 100-200μCi ^{113m}In-transferrin / animal following a 1:1 (v/v) labelling procedure. Other studies using ^{113m}In-transferrin as a marker have not indicated the ratio of isotope to plasma or whole blood required to obtain satisfactory binding (Hosain *et. al.*, 1969; Sisson & Oldendorf, 1971). Investigation of the optimal ratio for maximum binding may have improved the final vascular correction factor achieved with the tracer. However, if as indicated a substantial increase in plasma volume was required, the final quantity of injectate may have proved inappropriate for *in vivo* administration.

Interestingly, the transferrin molecule may not be ideally suited as a control marker in CNS tissue studies. The brain has a large requirement for iron (Pollitt & Leibel, 1982) and a high density of transferrin receptors has been reported on the luminal surface of neurovascular endothelial cells by immunostaining experiments (Friden, *et. al.*, 1991). Therefore, at CNS locations transferrin may be diverted from the blood, into the endothelium to deliver iron, to a greater extent than at other vascular sites. Furthermore, as part of a cerebrovascular transport system, changes in receptor density may exist as part of BBB dysfunction during EAE. Of note is the trend towards an increased ^{111}In-transferrin correction value in diseased animals compared to data from normal animals, observed during the study. Implementation of radiolabelled RBC as the blood volume marker did not indicate variation between normal and disease correction values.

RBC labelling provided a method by which 100% binding could be ensured. Furthermore, RBC are not involved in EAE disease pathology and there are no reports showing that RBC activity changes as a result of disease. The correction of ^{125}I BE values for blood volume achieved using labelled RBC compared well with the normal range reported in the original study by Leibowitz & Kennedy (1972).

By using ^{125}I-RSA and ^{111}In-RBC in combination differences between normal and disease states were consistently observed. Measurements of BBB permeability in EAE animals were in agreement with those of Leibowitz & Kennedy (1972) and Rumjanek *et. al.* (1984), who used similar regimes. Furthermore, the limited variation within normal tissue groups consistently found by Leibowitz & Kennedy (1972), Rumjanek *et. al.* (1984) and Butter *et. al.* (1991) has now been routinely demonstrated by the modified technique in the Lewis rat. Normal EVBE values obtained by Leibowitz & Kennedy (1972) were generally lower than figures reported here. However, Butter *et. al.* (1991) expressed similar baseline figures, suggesting that any variability may be due to experimental procedure.

The CNS tissues, C MP and CSp, selected for routine analysis include high and low lesion densities at the height of disease (Bolton *et. al.* ,1984b). Furthermore, the chosen areas display a range of neurovascular permeabilities to plasma protein as determined by the modified double radioisotope technique.

From published methodology a robust technique for the measurement of BBB permeability to plasma protein has been established. The method is highly reproducible and recognises a large difference between normal and EAE conditions. Therefore, the technique is suitable for detecting changes in abnormal neurovascular leakage following the administration of standard and novel drugs.

4.
Characterisation of BBB Dysfunction during the Course of Acute EAE in the Lewis Rat

4.1 Results

4.1.1 Profile of Neurovascular Breakdown during Acute EAE

Following the development of a reproducible method for the measurement of neuroantigen-induced BBB permeability, it was necessary to perform a series of studies to characterise the breakdown of normal neurovascular function during the course of acute EAE. The Lewis rat model of EAE has been fully characterised in terms of the development of disease symptoms (Paterson, 1976). However, body weight and neurological scores were observed daily to enable direct comparison between the development of these parameters and BBB permeability.

4.1.1.1 Body Weight Changes and Development of Neurological Deficits

Typically both EAE and CFA-inoculated animals demonstrate a small weight loss immediately following sensitisation. Subsequently, CFA-inoculated rats gain weight at a rate comparable to normal uninoculated animals. EAE-sensitised rats initially accrue weight at a normal rate, but a marked weight loss is noted around D10 ± 1 PI immediately prior to the onset of early neurological signs (Figure 21a). Weight loss continues rapidly over the next 8 ± 1 days while disease symptoms are apparent (Figures 21b & 22). Typically the height of disease occurs at D14 PI (Figure 22) afterwhich the neurological deficits begin to resolve. Weight gain resumes 2 - 3 days after all neurological deficits have disappeared.

4.1.1.2 BBB Dysfunction

Analysis of BBB permeability in CFA-inoculated controls was performed at a range of timepoints from D0 - D35PI (Figure 23). No significant elevation of BBB permeability due to CFA-inoculation was noted at any time following sensitisation in the CNS tissues examined. Protein extravasation across the neurovasculature at timepoints 10 and 23 PI was seemingly raised in 2 or 3 tissues. However, the elevation corresponded to a high baseline and values were not found to be significantly different from normal controls analysed in the same experiment. These

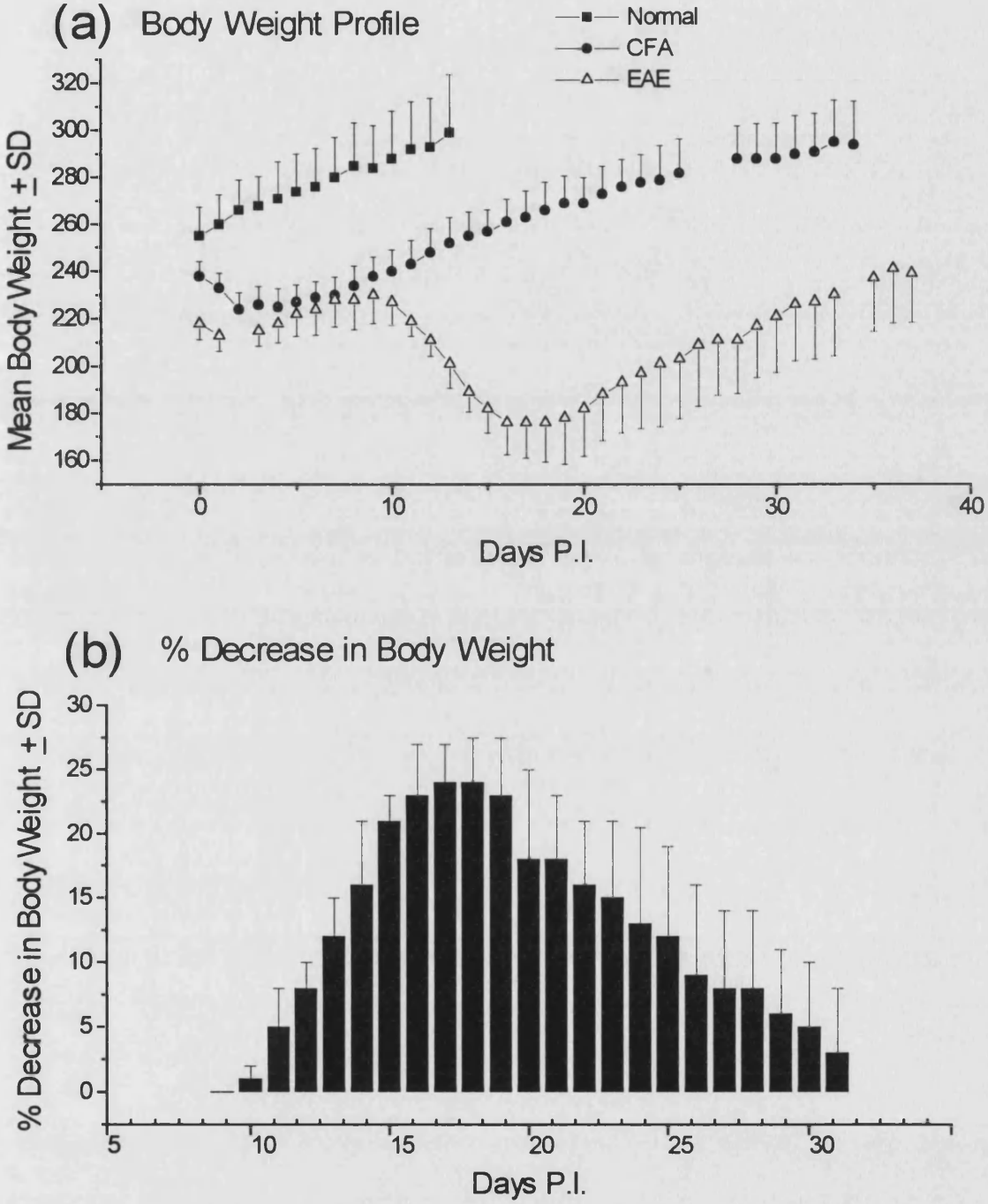


Figure 21: Body Weight Profiles for EAE and CFA-inoculated Lewis Rats with Comparison to Normals. **(a)** Groups of animals were weighed daily and mean body weights plotted against day number PI. Profiles shown for: normal group recorded for 14 consecutive days —■— (n = 6); CFA group recorded from D0 PI to D35 PI —●— (n = 4); EAE group recorded from D0 PI to D37 PI —Δ— (n = 6). **(b)** The percentage decrease in body weight from the peak body weight at D9-10 P.I. was calculated for EAE-sensitised animals (n = 6).

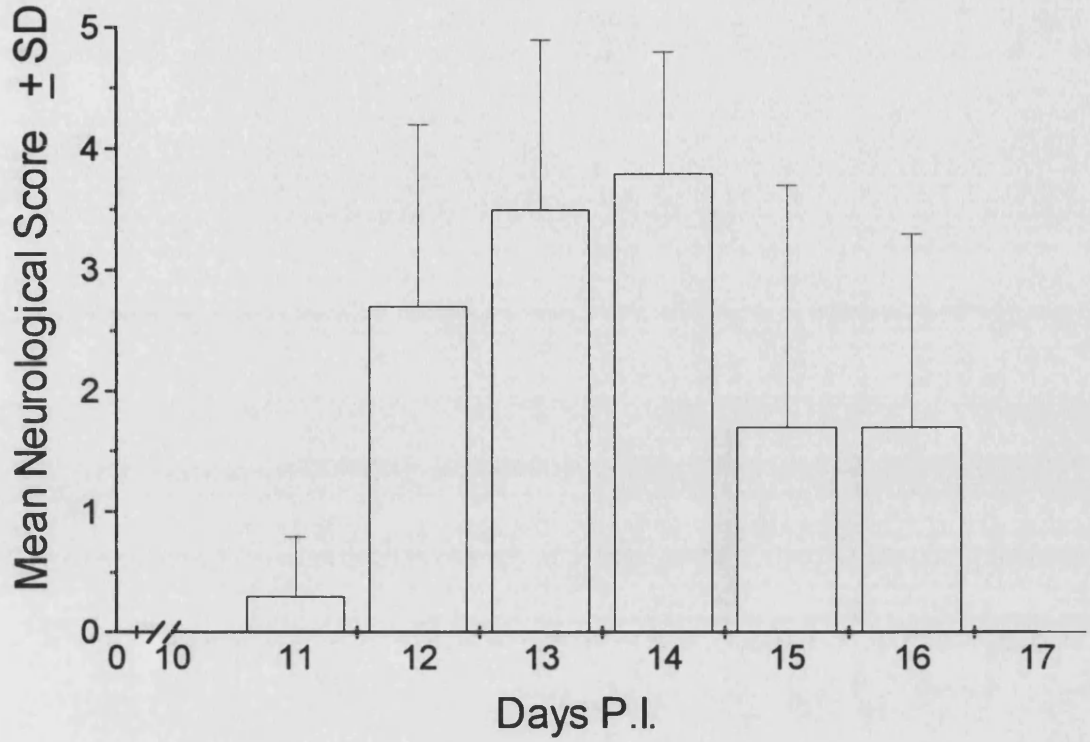


Figure 22: Development of Neurological Symptoms in EAE-sensitised Lewis Rats. Animals inoculated for EAE were weighed daily and assessed for disease symptoms subsequent to observed weight loss on days 9-11 PI (n = 6).

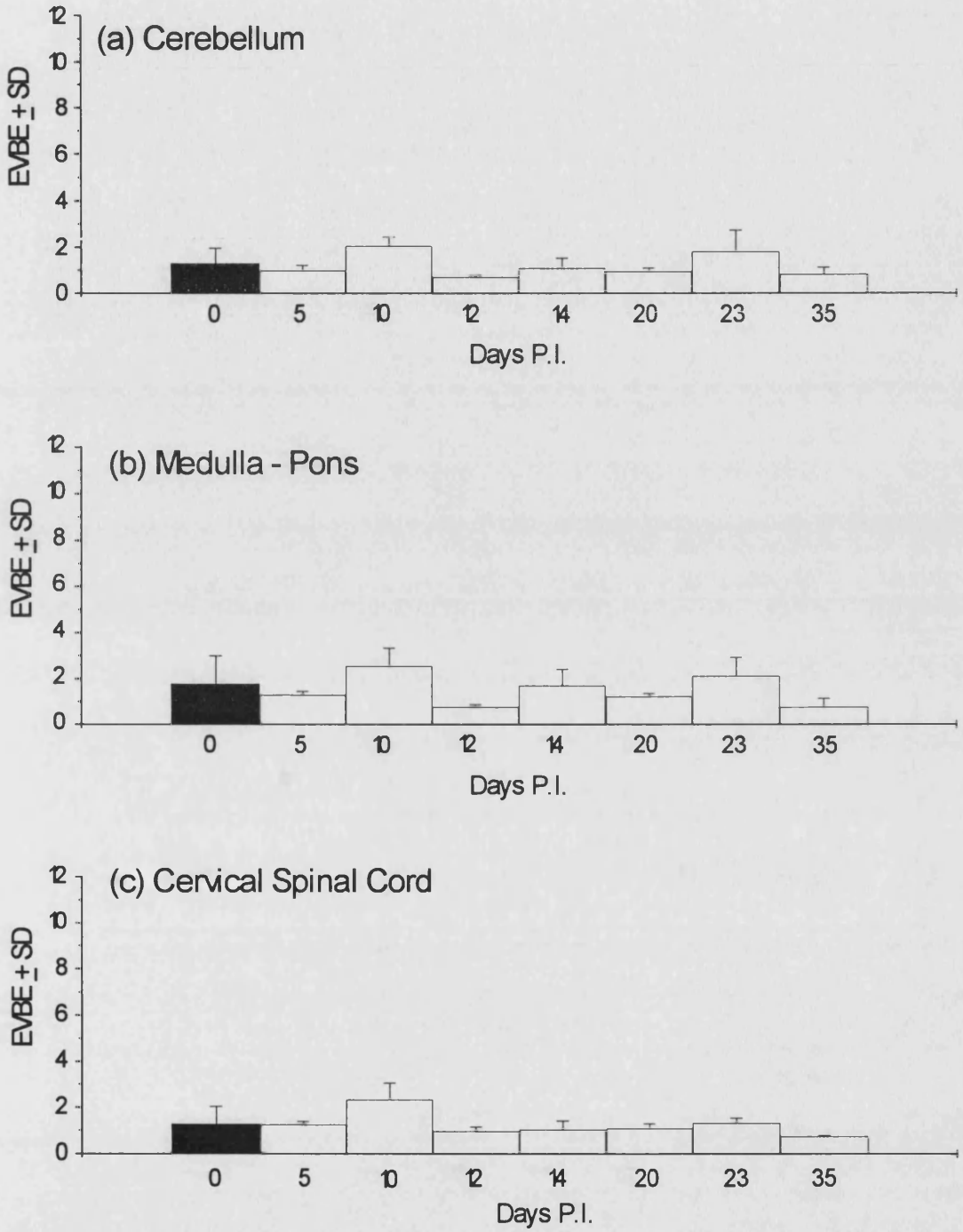


Figure 23: Profile of Protein Extravasation in the CNS of CFA-inoculated Lewis Rats. ^{125}I-RSA accumulation within the **(a)** cerebellum, **(b)** medulla-pons and **(c)** cervical spinal cord tissues, was measured in CFA-inoculated animals at a range of timepoints PI. The histograms combine the data from four experiments. The normal column (black) combines the normal values from all four experiments thereby presenting a full range of normal results (n = 14). CFA-inoculated animals were sampled at days 5 PI (n = 5), 10 PI (n = 5), 12 PI (n = 5), 14 PI (n = 5), 20 PI (n = 4), 23 PI (n = 4) and 35 PI (n = 5). The Mann-Whitney U test with Bonferroni correction for multiple comparisons found no significant differences between normal and CFA groups. Individual CFA groups were compared to the normal controls corresponding to the original experiment and not to the combined value plotted above.

results demonstrate that a non-specific increase in BBB permeability to protein does not occur. Therefore, any significant elevations in neurovascular dysfunction measured in EAE-sensitised animals will result from neuroantigen-mediated effects.

BBB dysfunction during EAE was examined in the C, MP and CSp tissues at timepoints D0, 5, 10, 11, 12, 15, 23 and 35-7 PI. In all tissue areas BBB permeability to protein remained within normal limits at D5 PI (Figure 24). At the time of weight loss the integrity of the BBB was maintained and although the EVBE values appear raised in relation to the combined normal results, no significant elevation was noted compared to normal and CFA controls run simultaneously. Neurovascular permeability is raised but not significantly in MP and CSp tissues at D11 PI, corresponding with symptom onset. The BBB within the MP and CSp tissues reachs a maximum permeability at D12PI (CSp D12 PI 8.3 ± 2.9 compared to D0 PI 1.35 ± 0.66; $p<0.05$). However, while BBB dysfunction in the C is significant at D12 PI ($p<0.05$) a marked protein extravasation does not develop until D15 PI. At D15 PI, the MP maintains the BBB dysfunction seen on D12 , but the CSp barrier permeability is reduced.

Analysis of the early recovery phase (D23 PI), following loss of neurological deficits and resumed weight gain, presents an unexpected tissue profile of neurovascular breakdown (Figure 24). Interestingly, the neurovasculature remains abnormally permeable to albumin in all CNS areas examined (C and CSp $p<0.1$, MP $p<0.05$). Furthermore, the C demonstrates BBB dysfunction greater than that seen during the acute phase (D23 PI, 7.61 ± 4.66; D12 PI, 2.18 ± 0.47), while MP and CSp tissues have reduced EVBE values (D23 PI - 6.32 ± 3.77, D12 PI - 8.4 ± 2.77; and D23 PI - 4.8 ± 1.98, D12 PI - 8.3 ± 2.89 respectively).

Examination of CNS tissues at D35-37 PI during the late recovery phase demonstrated a return to normal levels of BBB permeability to plasma protein in MP and CSp tissues and a marked reduction in dysfunction in the C (C, $p = 0.032$; MP, $p = 1.00$; CSp, $p = 0.124$; compared to normal).

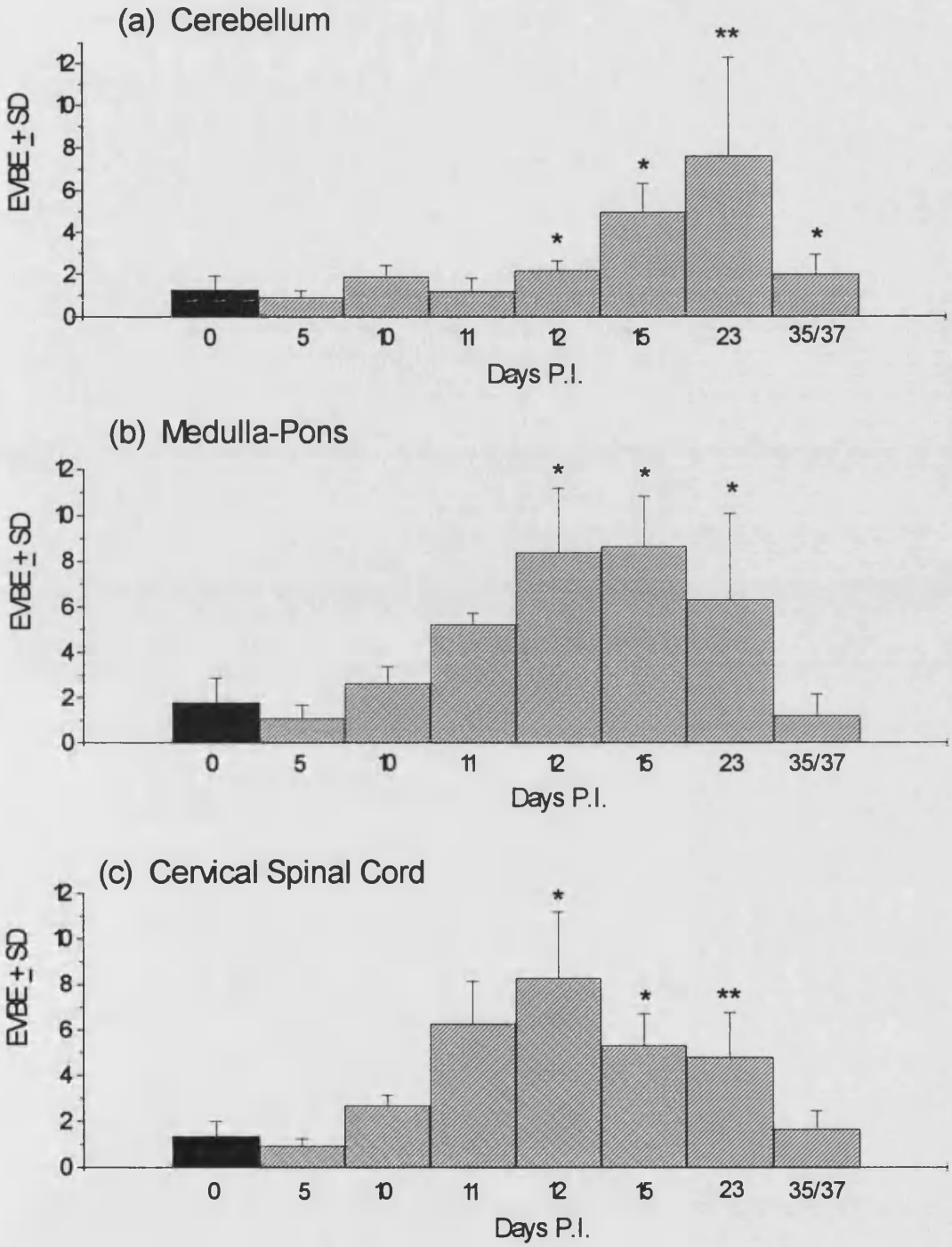


Figure 24: Profile of Protein Extravasation in the CNS during the Development of Acute EAE in the Lewis Rat. ^{125}I-RSA accumulation within the **(a)** cerebellum, **(b)** medulla-pons and **(c)** cervical spinal cord tissues, was measured in EAE-inoculated animals at a range of timepoints PI. The histograms combine the findings of five experiments. The normal column (black) combines the normal values from all five experiments thereby presenting a full range of normal results (n = 17). EAE-sensitised animals were sampled at days 5 PI (n = 6), 10 PI (n = 5), 11 PI (n = 3), 12 PI (n = 7), 15 PI (n = 4), 23 PI (n = 9) and 35-37 PI (n = 8). Statistical analysis of differences from normal BBB permeability was performed using the Mann-Whitney U test with Bonferroni correction for multiple comparisons; * $p < 0.05$, ** $p < 0.01$. Individual EAE groups were compared to the normal controls corresponding to the original experiment and not to the combined value plotted above.

4.1.2 Neurovascular Dysfunction: Comparison of Routes of Vehicle Administration

The pharmacological studies reported in later sections employ a variety of drug administration routes. As the effect of different regimes on abnormal BBB permeability is unknown a comparison of results from oral, i.p. and s.c. routes employing PBS alone was made early in the examination of drug studies.

EVBE values were elevated at D12 PI for all EAE-sensitised treatment groups and showed greatest neurovascular permeability in the MP and CSp areas as found in the tissues of untreated diseased animals (Figure 25). Furthermore, examination of the data by ANOVA demonstrated no significant difference between treatments at $p<0.05$.

4.1.3 Neurovascular Dysfunction: Correlation with Neurological Signs

Subsequent to the pharmacological studies performed in the acute model of EAE and reported in the following chapters, a comprehensive review of the extent of BBB dysfunction and the presentation of disease symptoms was undertaken (Figure 26a-c). Interestingly, the results demonstrate an absence of significant differences between any neurological grouping of EVBE results confirmed by the failure to show a linear regression between the two parameters in C or MP tissues. A minor trend towards increasing permeability with intensifying severity of disease is indicated in the CSp, but only 8% of the variation in results in the tissue fell within the 95% confidence limits of the regression. The data indicates that a full range of EVBE values can be obtained at both weight loss (C 0.98 to 10.66; CSp 1.33 to 16.48) and at the height of disease (CHLP: C 1.67 to 9.12; CSp 3.24 to 17.29). Consequently the neurological deficits displayed do not appear to be indicative of the extent of neurovascular breakdown.

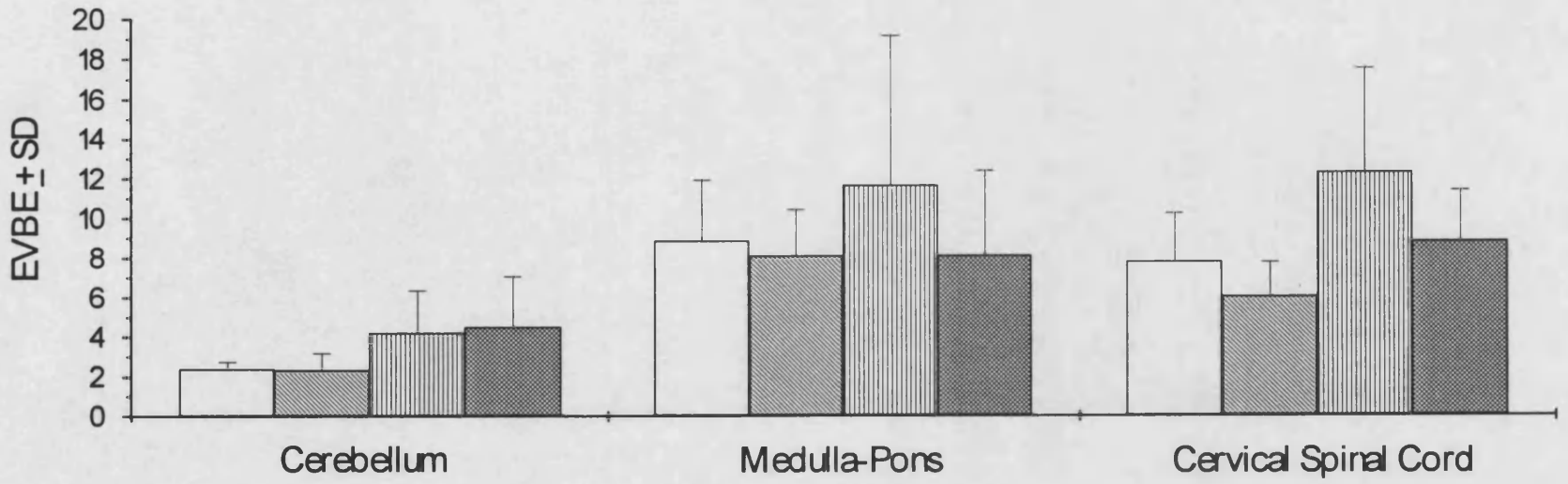
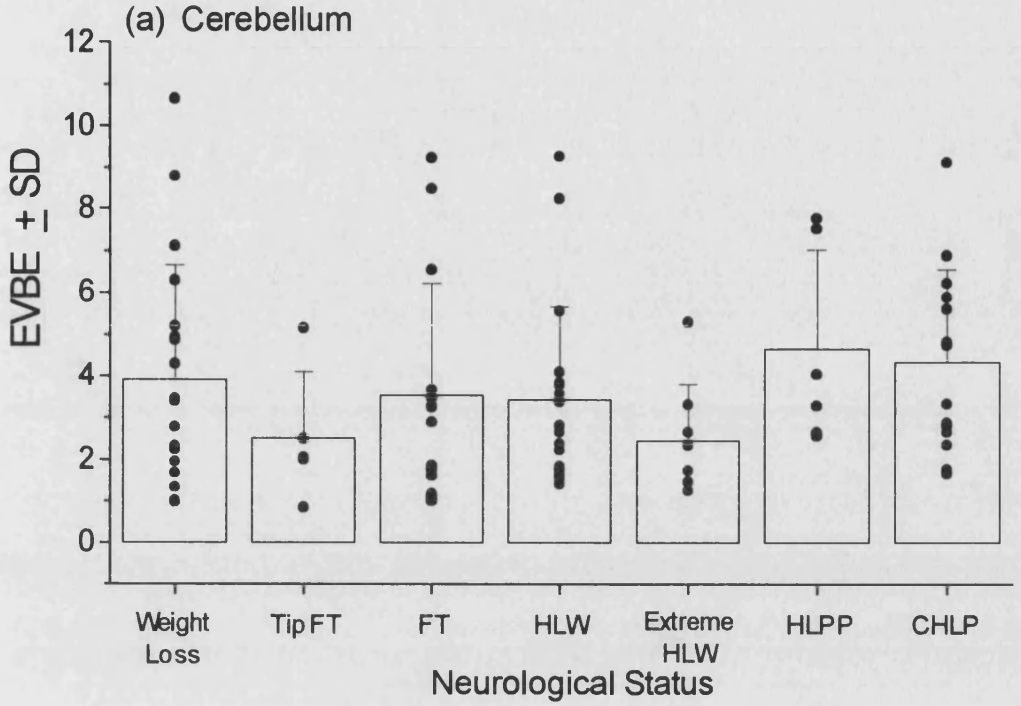
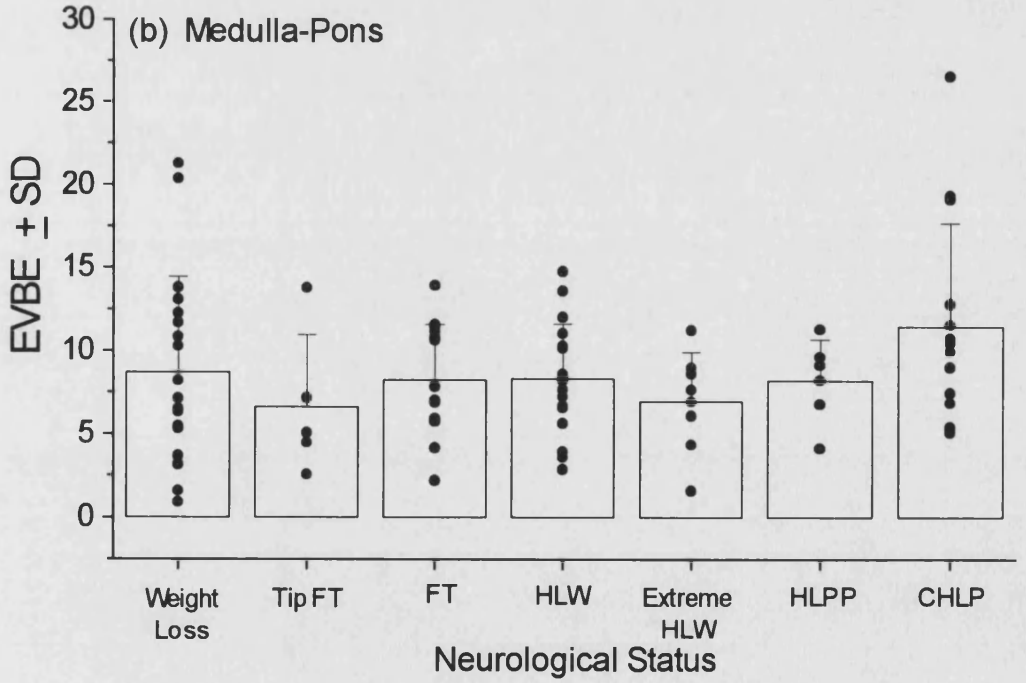


Figure 25: Route of Vehicle Administration: Effect on the Permeability of the BBB to Protein

Animals inoculated for EAE were dosed with sterile PBS via three different routes according to the therapeutic dosing regime for the acute phase (Section 2.7). The histogram compares oral (diagonal hatch column; n = 7), s.c. (vertical hatch column; n = 6) and i.p. (cross hatch column; n = 7) administration of PBS from separate experiments with an untreated time-matched EAE-sensitised control (open column; n = 5). Dosing began on the day of weight loss and continued for two days, dosing once daily for oral and i.p. administrations and twice daily for the s.c. regime. Animals were sacrificed on the day after the end of treatment. No significant difference was shown between groups by ANOVA.


(a) Cerebellum
EVBE +I SD
12
10
8
6
4
2
0
Weight Loss
Tip FT
FT
HLW
Extreme HLW
HLPP
CHLP
Neurological Status


(b) Medulla-Pons
EVBE +I SD
30
25
20
15
10
5
0
Weight Loss
Tip FT
FT
HLW
Extreme HLW
HLPP
CHLP
Neurological Status

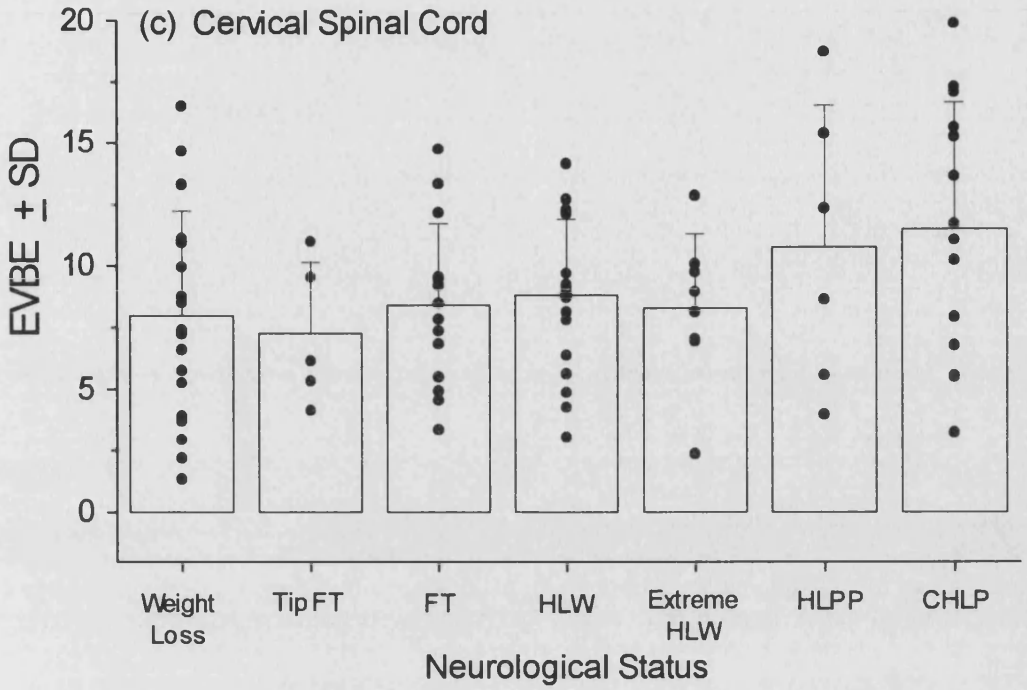


Figure 26: Correlation between Neurovascular dysfunction and Severity of Neurological Deficits in EAE-sensitised Lewis Rats.

A comprehensive review of the EAE-sensitised vehicle control groups from drug investigations has provided the data for this study. Three areas of the CNS have been assessed **(a)** cerebellum, **(b)** medulla-pons and **(c)** cervical spinal cord. Individual EVBE values are assigned to the symptom of greatest severity observed in the corresponding animal at the time of sampling. Individual EVBE values are shown by filled circles, while the histograms represent the mean EVBE ± SD for each neurological state. Statistical analysis using a one-way ANOVA shows that no difference exists between EVBE groupings in any tissue. Furthermore, no significant regression was demonstrated for C and MP tissues with only a minor regression accounting for 8% of the variation in results noted for the CSp.

4.2 Discussion

The double radioisotope technique for the determination of protein extravasation across the BBB, demonstrated a clear profile of elevated neurovascular dysfunction during the course of EAE. The extent of permeability enhancement compared well with profiles previously published for EAE in the guinea-pig (Leibowitz, 1969; Leibowitz & Kennedy, 1972), Biozzi mouse (Butter *et. al.*, 1991) and the Lewis rat (Rumjanek *et. al.*, 1984a; Lam, 1986). Furthermore, characterisation of body weight profiles and neurological scores demonstrate a typical progression of acute disease (Bolton & Flower, 1989).

A study of BBB permeability in CFA controls clearly demonstrated the absence of non-specific protein extravasation at the neurovasculature at any stage PI in the acute Lewis rat model. This is in agreement with the double radioisotope assessment of barrier dysfunction by Butter *et. al.* (1991). However, the finding is in contradiction to the observations of Reiber and Suckling who, by a determination of CSF protein content , found CFA to influence normal barrier function at D5-7PI (Reiber *et. al.*, 1984; Suckling *et. al.*, 1984). The disagreement between findings may be due to the methods employed as Reiber and Suckling analyse CSF composition which is more applicable to the study of blood-CSF barrier function than to BBB investigations.

Neurovascular abnormalities in EAE-sensitised rats were not observed prior to the onset of neurological deficits in the current study. Similarly, tissue uptake studies using large radiolabelled tracers have not demonstrated abnormal BBB permeability prior to symptom onset for either albumin (MW 60 000Da) or dextran (MW 60-90 000Da) (Leibowitz & Kennedy, 1972; Oldendorf & Towner, 1974; Lam, 1986). However, before development of disease symptoms smaller tracers such as mannitol (MW 182Da), DTPA (MW 550Da) and inulin (MW 5500Da) may be detected in the CNS compartment at abnormal levels (Oldendorf & Towner, 1974;

Daniel *et. al.*, 1981; Hawkins *et. al.*, 1991). Whether neurovascular damage could be detected by smaller molecules at a pre-disease timepoint during the Lewis model of acute EAE was not addressed by the present study.

Interestingly, Traugott, (1989) detected albumin deposits at timepoints prior to D9PI in acute EAE, by immunocytochemical analysis of frozen CNS sections. The technique enabled small focal areas of protein to be identified which would not have influenced an assessment of whole tissue albumin uptake. The importance of small protein and immunoglobulin deposits within the CNS prior to disease onset is unclear. However, as this coincides with a low grade increase in T cell surveillance of CNS tissue, the protein detected may be extravasating together with the penetrating cells (Traugott, 1989).

Increased neurovascular permeability was typically observed one day post-weight loss, concomitant with the onset of neurological deficits. These findings support those of Leibowitz (1969) and Leibowitz & Kennedy (1972) in the acute guinea pig model, and the MRI analysis by Seeldrayers (1993) in adoptively transferred EAE. Analysis of BBB dysfunction by other groups was evaluated by severity of disease and not day number PI, obviating direct comparison of barrier abnormalities. However, all models of EAE demonstrate elevated protein extravasation during the expression of symptoms (Rumjanek *et. al.*, 1984a; Butter *et. al.*, 1991; Hawkins *et. al.*, 1991). Indeed, in the present study, neurovascular permeability increased as the disease progressed.

An interesting and consistent aspect of the acute EAE profile was the continuance of BBB disruption in early recovery for all tissues studied following the loss of neurological deficits. Rumjanek *et. al.* (1984a) also observed barrier abnormalities in the brain tissue of convalescent animals. However, other studies employing isotopic tracers (Cutler *et. al.*, 1967; Juhler *et. al.*, 1984) and MRI analysis (Hawkins *et. al.*, 1991) in acute EAE, failed to demonstrate a continued BBB dysfunction. Resolution of disease abnormalities may occur at different rates

according to inoculum and species employed, accounting for the conflicting reports of neurovascular disruption during recovery.

The extent of BBB permeability in different CNS tissues was noted to change between acute and recovery stages of EAE. During disease onset the CSp and MP displayed markedly greater protein extravasation than the C. However, when analysed during early recovery following loss of all neurological deficits the vasculature of the C was disrupted to a greater extent than the brainstem and spinal cord regions. Interestingly, this profile correlates with the distal development of inflammatory lesions (Juhler *et. al.*, 1984; Bolton *et. al.*, 1984b). Furthermore, Rumjanek *et. al.* (1984a) also observed greater vascular permeability in the spinal cord during onset and in the brain during recovery. A predilection for elevated neurovascular permeability in spinal cord regions during the acute phase of EAE has been a common finding in many tracer studies (Oldendorf & Towner, 1974; Simmons *et. al.*, 1982; Butter *et. al.*, 1991).

The inference from onset and height of disease measurements of protein extravasation is that neurovascular abnormalities increase according to disease severity (Leibowitz & Kennedy, 1972; Rumjanek *et. al.*, 1984a; Butter *et. al.*, 1991). However, it is clear from analysis of results in the present investigation that no correlation exists between EVBE and neurological score. Once BBB dysfunction is triggered, individual animals may express different levels of elevated cerebrovascular permeability. Alternatively, the length of time since the loss of neurovascular integrity could be linked with increased tracer extravasation. Interestingly, in agreement with the earlier correlation between lesion distribution and tissue vascular permeability, Hawkins *et. al.* (1991) found a linear relationship between the number of enhancing lesions by Gd-DTPA MRI and the severity of disease. The lack of correlation between BBB breakdown and the extent of disease expression is similar to the situation observed in MS. Brain imaging techniques have highlighted the disparity between symptoms and plaque geography (Tourtellotte *et. al.*, 1984) demonstrating

the abundance of clinically silent lesions (Engell 1989). Indeed, actively enhancing lesions, indicative of a dysfunctional neurovasculature, may be found at all times in the brain of MS patients even during remission and immediately following successful treatment (Willoughby *et. al.*, 1989; Kesselring, 1989).

In summary, a comprehensive assessment of protein extravasation across the BBB during the course of EAE has demonstrated a marked increase in permeability above normal values during onset, height of disease and recovery phases. Analysis of several CNS regions has demonstrated that the extent of neurovascular dysfunction is dependent on the area under study and the timepoint during EAE when the measurements are recorded.

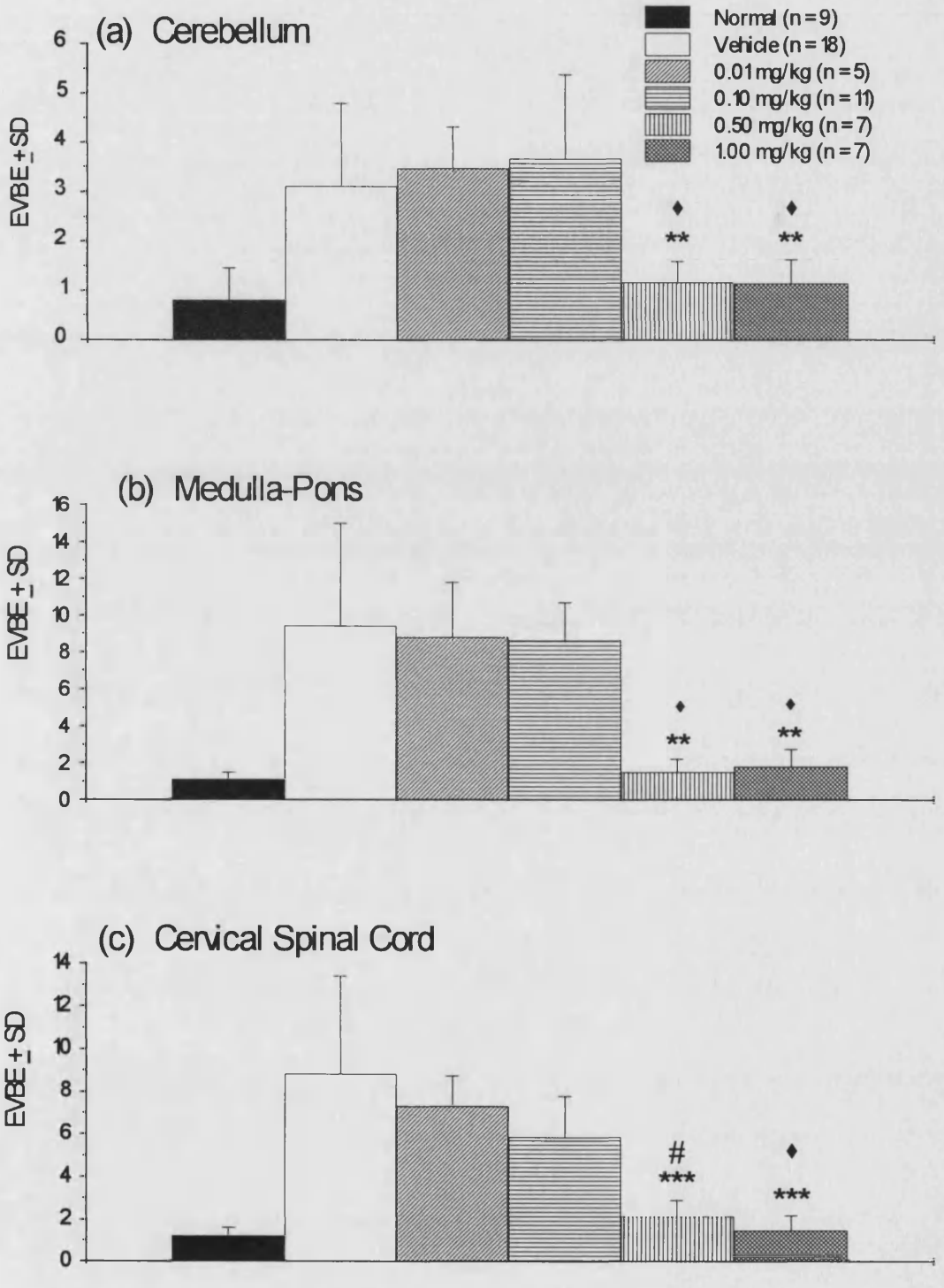
5.
Modulation of BBB Dysfunction by DEX Administration

5.1 Results

The use of DEX in EAE is well documented (Komarek & Dietrich, 1971; Desai & Barten, 1989; Bolton & Flower, 1989), but effects by the steroid on neuroantigen-induced BBB dysfunction has not been determined. However, glucocorticoid control of neurovascular function has been demonstrated in normal mice, experimental non-disease models of cerebro-microvascular abnormalities and indicated by reduced enhancing MRI scans following administration to MS patients (Hedley-Whyte & Hsu, 1986; Koenig *et. al.*, 1989b; Barkhof *et. al.*, 1991). DEX was therefore employed as a standard agent for analysing pharmacological control of abnormal BBB function in EAE.

5.1.1 Therapeutic Administration of DEX in the Acute Phase of Disease

To achieve a determination of glucocorticoid function of relevance to the therapy of MS, a therapeutic dosing regime was chosen for study. Dosing began on the first day of weight loss, approximately D10 PI, and continued for two days. The effect of DEX at concentrations between 0.01 and 1.0mg/kg body weight on development of abnormal neurovascular permeability in the acute phase of EAE was studied over the course of three experiments and the results are detailed in Figure 27a-c. Low dose treatment (0.01mg/kg body weight) failed to modify neurovascular permeability in the CNS tissues studied or to influence the final MNS (Table 5). A ten-fold increase in concentration (0.1mg/kg body weight) produced an improvement in barrier function in the CSp, which was complemented by a reduced MNS ($p < 0.05$). Increasing the dose to 1.0mg/kg body weight completely prevented neurovascular dysfunction in all sampled tissues (C, MP $p < 0.01$; CSp, $p < 0.001$). A lower steroid dose of 0.5mg/kg DEX significantly reduced the microvascular dysfunction to the same degree as 1.0mg/kg, but was found to be significantly


(a) Cerebellum
Normal (n=9)
Vehicle (n=18)
0.01 mg/kg (n=5)
0.10 mg/kg (n=11)
0.50 mg/kg (n=7)
1.00 mg/kg (n=7)
EVBE+SD
0
1
2
3
4
5
6
♦
**
♦
**
(b) Medulla-Pons
EVBE+SD
0
2
4
6
8
10
12
14
16
♦
**
♦
**
(c) Cervical Spinal Cord
EVBE+SD
0
2
4
6
8
10
12
14
#
***
♦
***

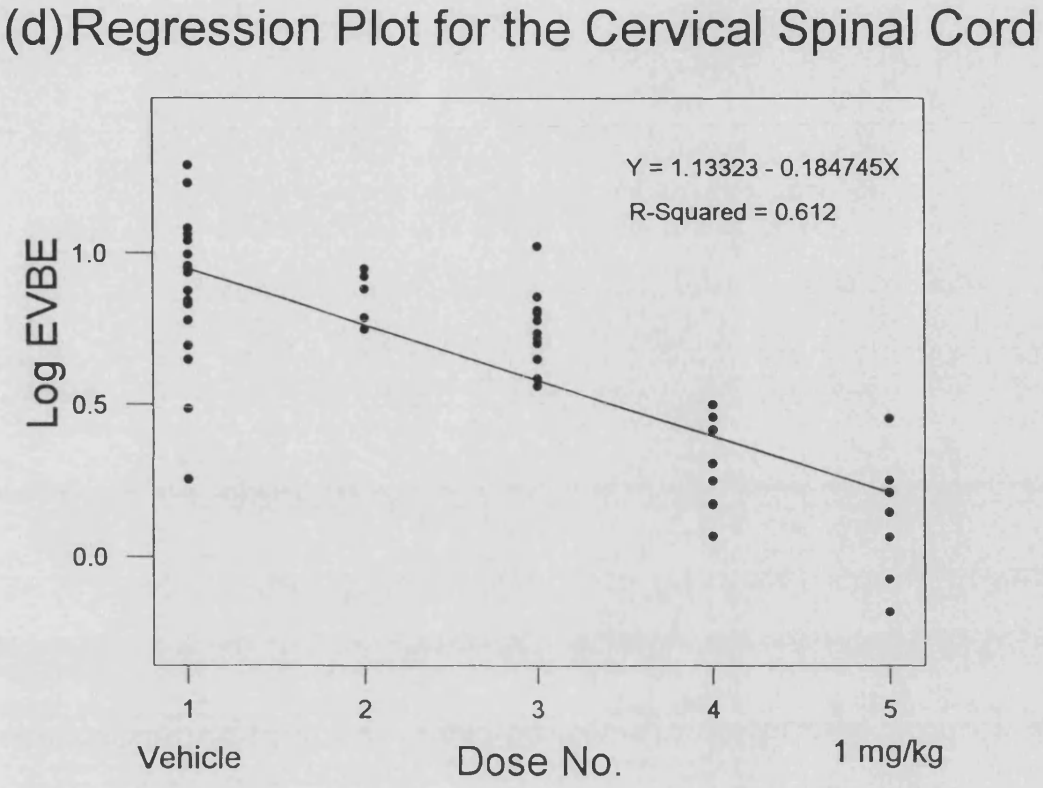


Figure 27: Inhibition of BBB breakdown by DEX Administered During the Acute Phase of EAE. DEX was administered twice daily for two days beginning on the day of weight loss. BBB dysfunction was measured on the day following the conclusion of treatment in **(a)** cerebellum, **(b)** medulla-pons and **(c)** cervical spinal cord tissues. The treatment groups were pooled from three experiments. Normal animals (black column; n = 9), vehicle-dosed EAE controls (open column; n = 18), 0.01mg/kg DEX (diagonal hatch column; n = 5), 0.1mg/kg DEX (horizontal hatch column; n = 11), 0.5mg/kg DEX (vertical hatch column; n = 7) and 1.0mg/kg DEX (cross hatch column; n = 7). Statistical analysis of individual points used the Mann-Whitney U test with Bonferonni correction for multiple comparisons: ** $p < 0.01$ compared to vehicle control; *** $p < 0.001$ compared to vehicle control; ♦ no significant difference to normal group; # $p < 0.05$ compared to normal group. The reduction seen with DEX at 0.1mg/kg in the CSp was not significant following correction for multiple comparisons. Regression analysis of the log response showed a significant linear relationship in all tissues ($p = 0.0$). Figure **(d)** illustrates how closely the actual data for the CSp aligns with the fitted regression plot (61% fit).

different from normal in the CSp ($p < 0.05$). Both 1.0 and 0.5mg/kg doses demonstrated highly significant suppressive effects on the presentation of neurological deficits ($p < 0.001$; Table 5). The dose-dependent restriction of BBB opening by DEX was most apparent in the CSp and was reflected in the regression analysis which found a significant linear relationship and a 61% fit of data points (Figure 27d).

Histological examination of the CSp from animals treated with 0.1mg/kg DEX showed no significant effect on cellular infiltration (Table 5). However, high dose administration (1.0mg/kg DEX) markedly reduced the occurrence of perivascular cuffs ($p < 0.01$), but did not abolish lesion presentation.

Table 5: Summary of Neurological Status , Histological Scores and Plasma Corticosterone Levels following DEX Administration in the Acute Phase of EAE

	n	MNS ± SD	n	CSp Lesion no. ± SD	n	Corticosterone ng/ml ± SD
Normal	-	-	-	-	8	41.0 ± 9.7
D13 EAE[a]	8	1.63 ± 1.75	8	117.0 ± 61.0	-	-
Vehicle	19	2.76 ± 1.08	-	-	3	131.0 ± 39.6
0.01mg/kg DEX	5	2.80 ± 1.79	-	-	-	-
0.1 mg/kg DEX	9	1.33 ± 1.00*	5	99.6 ± 37.0	-	-
0.5 mg/kg DEX	7	0.14 ± 0.38#	-	-	6	10.8 ± 1.7
1.0 mg/kg DEX	7	0#	6	8.7 ± 5.0≠	-	-

* $p < 0.05$; ≠ $p < 0.01$; # $p < 0.001$

[a] Two days post-weight loss.

MNS was recorded on the day of sampling and results were analysed using ANOVA and Dunnett's multiple comparison to a single control. Lesion numbers relate to the total number of inflammatory cuffs seen per section of CSp; T-test comparison with EAE control. Endogenous corticosterone plasma concentrations analysed by Mann-Whitney U demonstrated DEX treatment at 0.5mg/kg to be significantly different from vehicle (*) and normal (≠) controls.

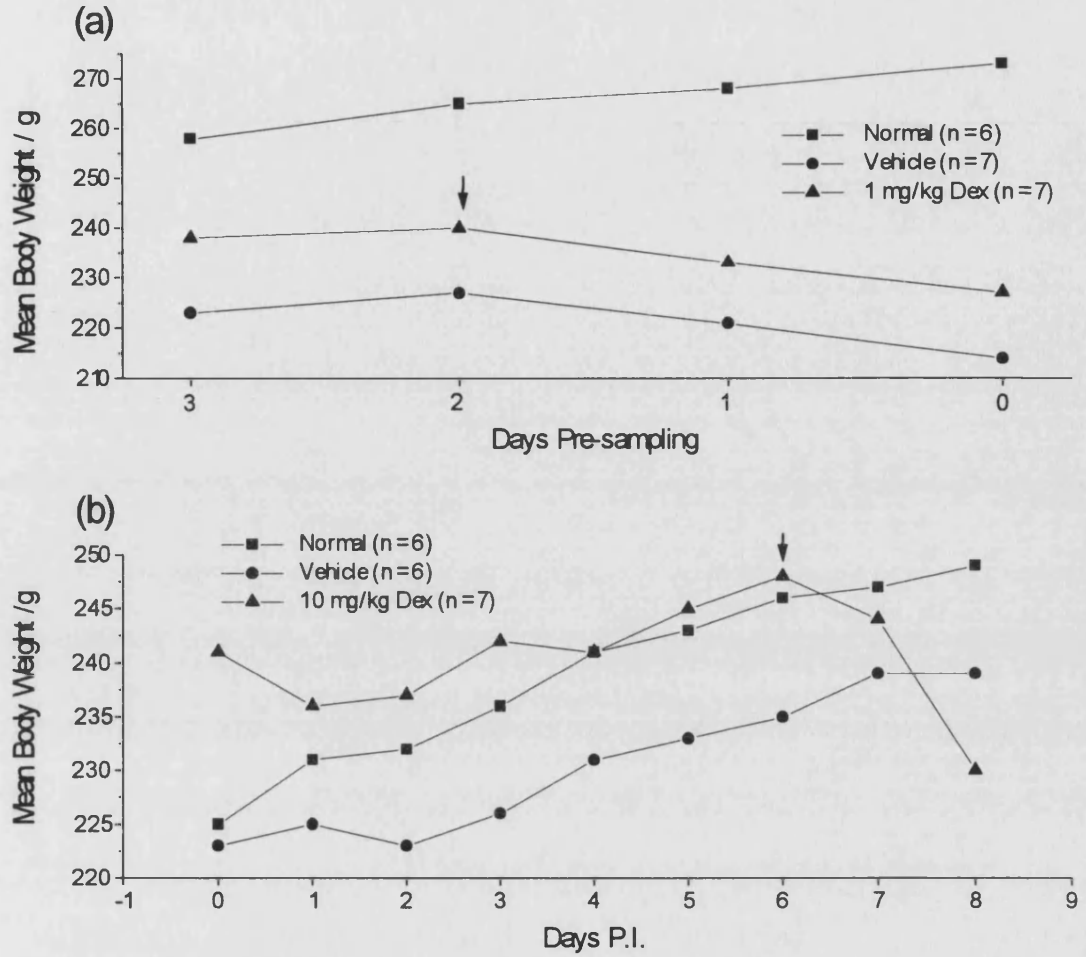
The endogenous corticosterone level in animals dosed with 0.5mg/kg body weight DEX was significantly reduced from vehicle control and normal baseline concentrations ($p < 0.05$ and 0.01 respectively), suggesting a typical negative feedback response (Table 5).

The doses of DEX used in the study did not affect body weight profiles during the dosing period (Figure 28a). Previous work had shown that a dose of 10mg/kg body weight DEX, given from D6 PI to D8 PI, initiated a dramatic weight loss in EAE-sensitised animals at a time when weight gain is typical (Figure 28b). No enhancement of weight loss above that normal for the progression of EAE in the acute phase was experienced at the highest dose of 1.0mg/kg body weight. Calculation of the mean percentage weight loss showed no significant difference between vehicle and drug treatments with the exception of the 0.01mg/kg group. However, this dose was ineffective in improving aspects of EAE and as significant weight loss was not experienced at higher concentrations of DEX no adverse drug effect was suspected.

5.1.2 Administration of DEX during the Early Recovery Phase of Disease

The initial study of BBB breakdown during EAE highlighted the continued neurovascular dysfunction during the early recovery phase. An investigation was undertaken to determine the effect of DEX on the continuing BBB disruption of the recovery phase, in the absence of symptoms.

The results in Figure 29 demonstrate an enhanced BBB permeability to radiolabelled protein in vehicle controls confirming previous observations in early recovery. A dose of 0.01mg/kg body weight DEX had no corrective effect on neurovascular function, which was comparable to the effect during the acute phase. Administration of 0.1mg/kg body weight DEX had shown a minor effect on the CSp tissue in the acute phase. However, during recovery a marked reduction of abnormal



(c)

	Vehicle[b]	0.01[c]	0.10	0.50	1.00
% Change in Body Weight[a]	5.9	9.7*	8.6	7.9	5.5
SD	2.3	2.1	2.7	2.0	2.1
n	7	5	6	7	7

[a] : % Body weight loss incurred between peak weight prior to symptom onset and the day of sampling

[b] : The vehicle treatment group was taken from the same experiment as 0.5mg/kg DEX and is representative of other vehicle treatment groups

[c] : Individual treatments include animals receiving a single batch of inoculum

Figure 28: Comparison of Weight Profiles and % Weight Loss in DEX and Vehicle Treated animals. Graph **(a)** shows the weight profiles of 1mg/kg DEX and vehicle treated EAE-sensitised animals. Treatment began on the day of weight loss (arrow) and continued for two days dosing twice daily. Sampling (0) occurred on the day following the end of treatment. Graph **(b)** shows the weight profiles of 10mg/kg DEX and vehicle treated EAE-sensitised animals compared to normals. Dosing was for two days dosing twice daily beginning on D6 PI (arrow) with sampling on D8 PI. Table **(c)** compares % change in weight between groups dosed from weight loss twice daily for two days within the range 0.01 to 1mg/kg DEX. Results were analysed by ANOVA and Dunnett's test; * $p < 0.05$ compared to vehicle control.

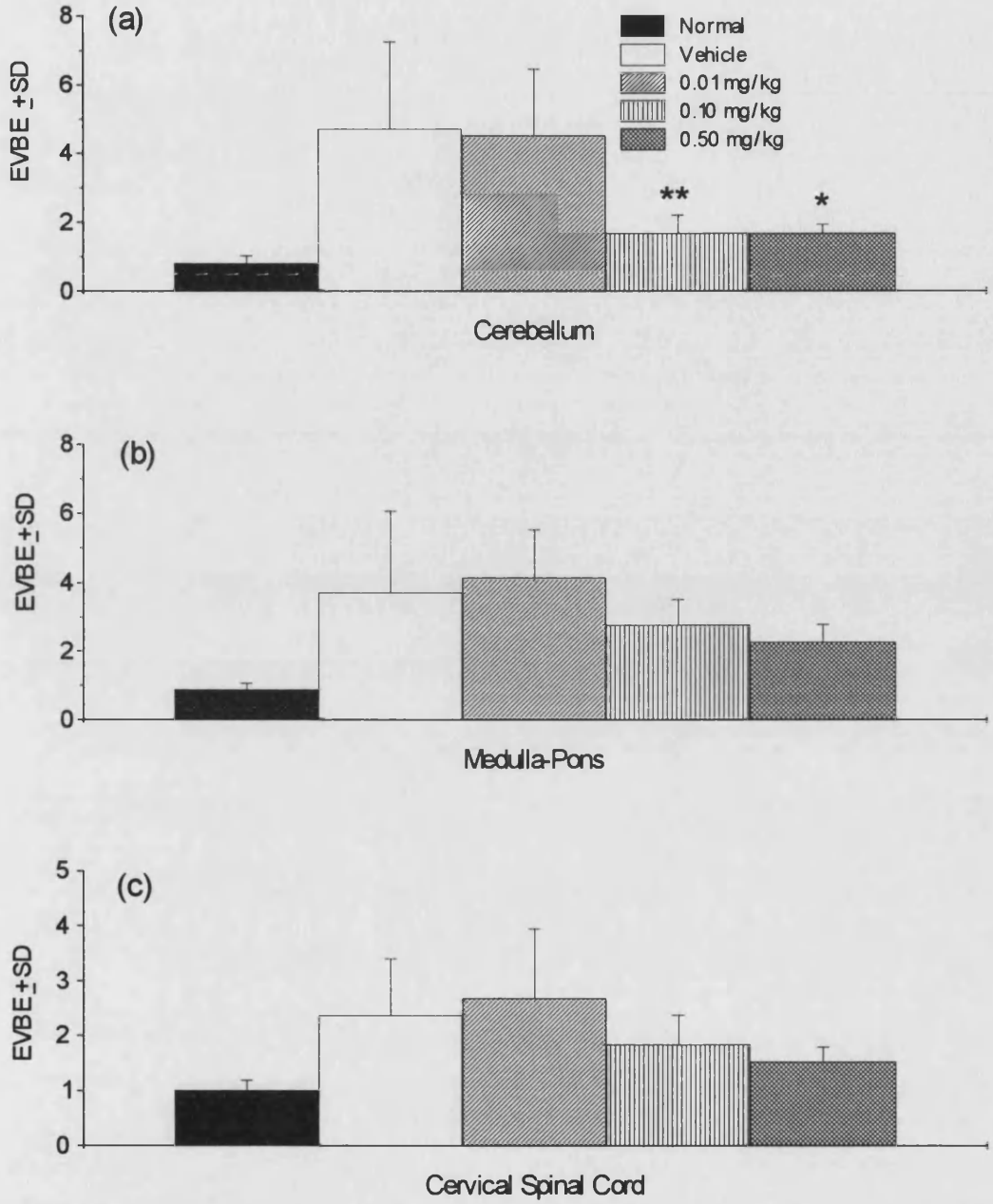


Figure 29: The Effect of DEX on Abnormal BBB Function during Early Recovery in the Acute Model of EAE. The results are compiled from two experiments and show profiles in **(a)** cerebellum, **(b)** medulla-pons and **(c)** cervical spinal cord tissues. Animals had shown a minimum neurological score of 2 during the acute phase of disease. Following the disappearance of signs and two consecutive days of weight gain the drug was administered twice daily for two days, sampling on the day following the final injection. Normals (black column; n = 6), vehicle control (open column; n = 8), 0.01mg/kg DEX (diagonal hatch column; n = 4), 0.1mg/kg DEX (vertical hatch column; n = 9) and 0.5mg/kg DEX (cross hatch column; n = 5). Significant differences to vehicle controls were determined by Mann-Whitney U with Bonferroni correction; * $p < 0.01$, ** $p < 0.01$. Regression of the log response against dose was significant in the C only ($p = 0.0$; MP, $p = 0.12$; CSp, $p = 0.07$).

BBB function in the C of EAE animals was demonstrated ($p < 0.01$). DEX at a dose of 0.5mg/kg body weight lowered the EVBE score in the MP and CSp and significantly in the C ($p < 0.05$). This is in contrast to the therapeutic effect of a 0.5mg/kg dose, which was highly significant in all tissues. A linear dose response was only shown in the C region of the CNS (regression; $p = 0.0$, 47% fit).

The sensitivity of BBB function to glucocorticoid control appears to vary between acute and early recovery phases of EAE. These results suggest that different mechanisms may be responsible for the abnormal extravasation of protein during the recovery stage of disease in the Lewis rat.

5.1.3 Reversal of DEX Control by Administration of the Steroid Antagonist RU 38486

To demonstrate that DEX was mediating an effect on BBB permeability through binding to the type II glucocorticoid receptor, two experiments were conducted using the steroid antagonist RU38486. Dosing of the antagonist was twice daily to obtain maximum efficacy (Bolton & Flower, 1989).

The glucocorticoid receptor antagonist was unable to significantly counteract the suppressive effect of 0.5mg/kg DEX on BBB permeability in any tissue when used at 20mg/kg, although the mean EVBE was raised in all tissue profiles (Figure 30). Furthermore, the neurological condition remained the same as DEX/vehicle controls (Table 6). Increasing the concentration of RU 38486 to 40mg/kg body weight, caused a significant reversal of the inhibitory effect of DEX on BBB dysfunction in all tissues ($p < 0.01$) and was within the vehicle/vehicle range of EVBE values. The neurological deficits of the 40mg/kg RU 38486 plus DEX group were also elevated, but no significant difference was demonstrated from the DEX/vehicle control, indicating that abolition of the DEX effect may not have been complete. Further analysis of the log response to RU 38486 doses showed significant linear regression in

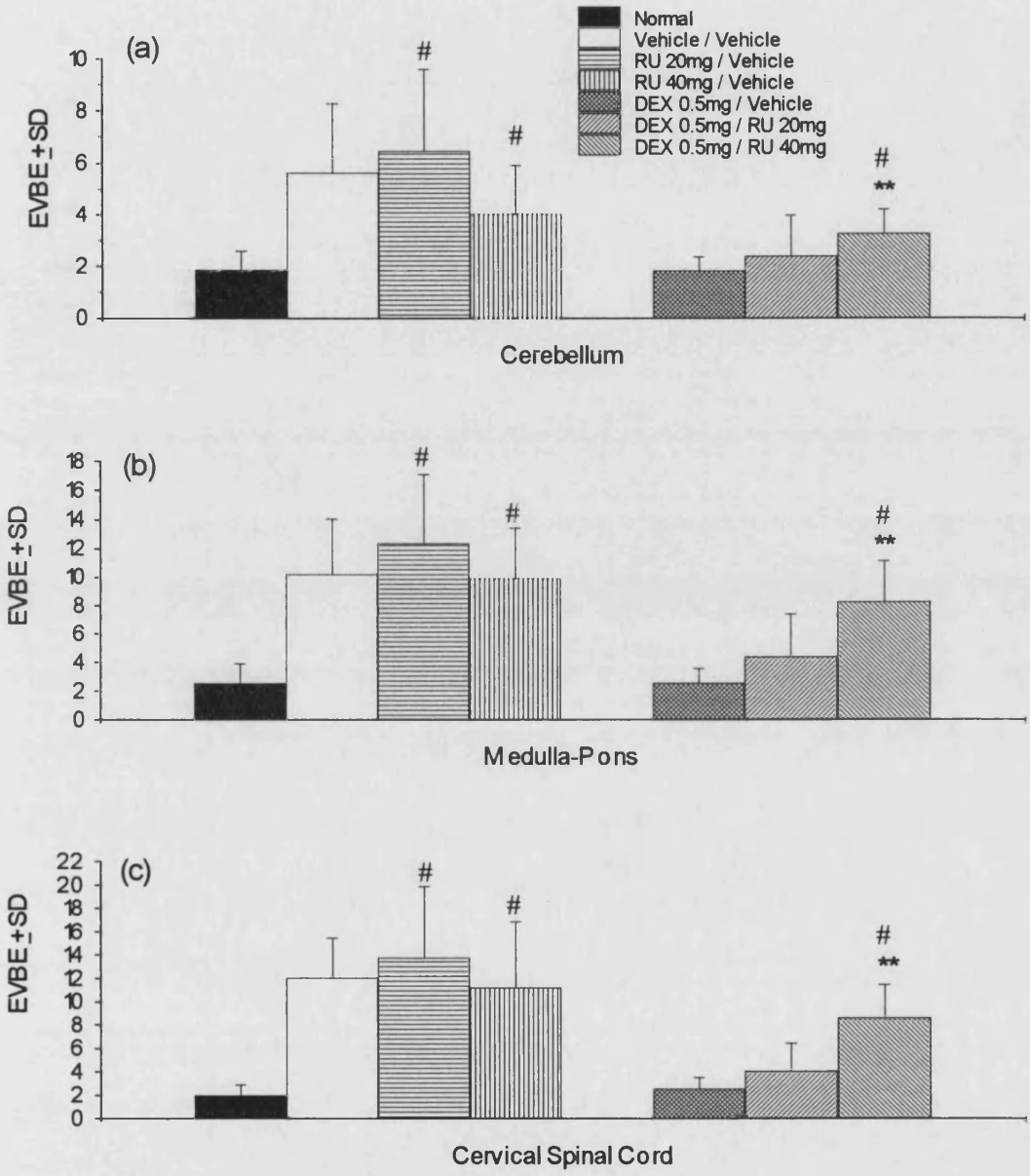


Figure 30: Effect of the Glucocorticoid Antagonist RU38486 on the Control of BBB permeability by DEX. RU38486 was employed at doses of 20 and 40 mg/kg to prevent the DEX-mediated suppression of abnormal BBB permeability to protein. The histogram combines the results of two experiments detailing three CNS areas **(a)** cerebellum, **(b)** medulla-pons and **(c)** cervical spinal cord from: normal (black column, n = 9); vehicle / vehicle (open column, n = 12); 20mg/kg RU38486 / vehicle (horizontal hatch column, n = 6); 40mg/kg RU38486 / vehicle (vertical hatch column, n =6); vehicle / 0.5mg/kg DEX (cross hatch column, n = 12); 20mg/kg RU38486 / 0.5mg/kg DEX (left-hand diagonal hatch column, n = 6); and 40mg/kg RU38486 / 0.5mg/kg DEX (right-hand diagonal hatch column, n = 6). # no significant difference from vehicle / vehicle, ** $p < 0.01$ significantly different from DEX / vehicle; Mann-Whitney U with Bonferroni correction for multiple comparisons. Log responses demonstrated significant linear regression against RU38486 concentration; C, $p = 0.005$, MP and CSp, $p = 0.0$ with 30 and 56% fits respectively.

all tissues with 30, 56 and 56% of response variation being attributed solely to dose in C, MP and CSp tissues respectively.

Examination of the effects of RU 38486 administered in the absence of DEX, did not demonstrate significant variation in barrier dysfunction from the EAE vehicle control (Figure 30). Neither did the compound cause significant alteration of disease symptoms (Table 6).

Table 6: Antagonism of DEX-mediated Suppression of EAE by RU38486: Summary of Neurological Status and Weight Loss

Group	n	MNS ± SD	%Weight Loss ± SD#
Vehicle / Vehicle	12	1.79 ± 1.54*	8.32 ± 2.85
RU 20mg/kg / Vehicle	6	2.33 ± 1.97*	8.32 ± 3.37
RU 40mg/kg / Vehicle	6	1.67 ± 1.47*	7.68 ± 4.66
Vehicle / DEX 0.5mg/kg	12	0.04 ± 0.14≠	10.83 ± 4.60
RU 20mg/kg / DEX 0.5mg/kg	6	0≠	8.73 ± 2.34
RU 40mg/kg / DEX 0.5mg/kg	6	0.75 ± 0.88	8.78 ± 1.02

The experimental observations made in the table complement the EVBE results shown in Figure 30. MNS represent symptoms of disease recorded on D12PI when sampled and were analysed by Dunnett's test post-ANOVA: * $p < 0.05$ significantly different to vehicle/DEX treatment; ≠ $p < 0.05$ significantly different to vehicle/vehicle control. # no significant difference found between % weight loss in groups: ANOVA.

5.2 Discussion

The findings presented clearly demonstrate a dose-related suppression of abnormal neurovascular function by DEX during EAE. Furthermore, the dose-dependent inhibition of clinical signs and reduction of histological lesions previously shown in EAE following prophylactic dosing regimes for DEX (Komarek & Dietrich, 1971; Desai & Barten, 1989; Bolton & Flower, 1989), has now been confirmed for short-term therapeutic administration.

Glucocorticoids readily penetrate the neurovasculature and may therefore reach and modify both the components comprising the BBB and the inflammatory cells within CNS lesions (Pardridge & Mietus, 1979). Previous studies of neurovascular integrity in healthy rats and mice demonstrated the ability of DEX to reduce permeability below normal levels for a range of circulating tracers (Hedley-Whyte & Hsu, 1986; Ziylan *et. al.*, 1988). Moreover, a loss of neurovascular control is experienced following removal of endogenous glucocorticoids by adrenalectomy, which may be overcome by administration of DEX (Long & Holaday, 1985). A role for both endogenous and exogenously applied glucocorticoids in the maintenance of an intact neuroendothelium is therefore indicated.

A number of models of experimentally-induced brain edema have reported successful resolution of abnormal CNS water accumulation and barrier function following steroid administration (Pappius, 1972; Herrman *et. al.*, 1972; Koeing *et. al.*, 1989b). The results reported here now extend the observations of glucocorticoid action at the BBB to acute EAE. Furthermore, the findings are in agreement with reports of resolving enhancing lesions following steroid treatment of MS (Sears *et. al.* ,1978 ; Troiano *et. al.* ,1987 ; Barkhof *et. al.* ,1991 ; Miller *et. al.* ,1992).

Particularly interesting was the observation that DEX did not apparently restore normal barrier function to the same extent when administered during the recovery phase. In the acute stage of disease DEX had successfully normalised barrier

function, particularly in the MP and CSp regions, whereas significant restoration of neurovascular integrity was only achieved in the C during early recovery. The histological profile of lesions shown by Bolton *et. al.* (1984b) corresponds to the tissue and time distribution noted here for the development of BBB permeability. Control of an inflammatory cell component may therefore be pivotal to the correction of barrier abnormality by DEX. Alternatively, the mechanisms causing neurovascular dysfunction during disease expression may differ from those mediating BBB perturbation during convalescence. Consideration of the endogenous steroid profile of untreated EAE-sensitised rats, shows that corticosterone levels increase gradually during the onset of neurological deficits, peaking at the height of disease (Mackenzie *et. al.*, 1989; MacPhee *et. al.*, 1989).While the peak in endogenous steroid production is considered to initiate a clinical recovery, it is now clear that the elevated corticosterone levels are insufficient to restore normal BBB function within 5-8 days of maximum output. Therefore, a glucocorticoid-insensitive neurovascular permeability may be implicated for the later stages of EAE disease.

DEX primarily acts through the intracytoplasmic type II glucocorticoid receptor although some affinity towards the mineralcorticoid receptor, type I, has been noted. The steroid-receptor complex translocates to nuclear binding sites where gene transcription is either up- or down-regulated, often interacting with other signalling pathways. The type II receptor is located throughout the rat CNS, but is absent from the circumventricular organs (Ahima *et. al.*, 1992). Most glia express the glucocorticoid receptor (Ahima *et. al.*, 1992; Jung-Testas *et. al.*, 1992) including the astrocyte, which influences the integrity of the BBB endothelium. Furthermore, DEX has been implicated in the control of cellular protein synthesis within the vascular endothelium during global ischaemia (Tosaki, 1985).

The anti-glucocorticoid RU38486 binds to the type II glucocorticoid receptor with a greater affinity than DEX, thereby antagonising steroid activity (Kawai *et. al.*, 1989). Administration of RU38486 in the present study was observed to dose-

dependently reverse the control over abnormal barrier opening imposed by short-term therapeutic dosing of DEX during acute EAE. Therefore, a type II receptor-steroid interaction, potentially involving control of gene transcription, is suggested for the action of DEX on BBB function during EAE. The concentration of RU38486 employed was greater than that used by Bolton & Flower (1989) to abrogate glucocorticoid effects during EAE. However, the previous study did not analyse short-term therapeutic treatment or the control of BBB function. Also, the current work has demonstrated that a higher steroid dose is required to significantly effect neuro-endothelial integrity than is needed to reduce neurological deficits.

The site and mechanism of action of DEX in the control of neurovascular permeability is unclear. DEX has multiple actions and the method of regulating endothelial cell permeability at the BBB may differ between normal and pathological conditions. Furthermore, the cause of aberrant neurovascular function may dictate the routes by which DEX may exert an effect. Two main areas of possible steroid action can be considered when manipulating barrier function in EAE. Firstly, suppression of immune system activation and regulation of neurovascular perturbator synthesis by immune cells may result in the indirect control of barrier function. Secondly, DEX is able to control BBB permeability under both normal and non-immune mediated pathological conditions suggesting a regulatory role directed at the cells comprising the BBB. The location of neurovascular action may be directly at the vascular endothelial cell (Cronstein *et. al.*, 1992)) or on cells in close regulatory contact such as the astrocyte or pericyte (Jung-Testas *et. al.*, 1992). *In vitro* DEX has been shown to inhibit astrocyte-influenced microvessel morphogenesis, an action blocked by the glucocorticoid antagonist cortexolone, but not to exert an effect on non-astrocyte induced capillary-like formation (Wolff *et. al.*, 1992).

The anti-inflammatory capabilities of glucocorticoids are well recognised. Redistribution of inflammatory cells and edematous material away from inflammatory sites clearly reduces the damage to tissue and reduces clinical symptoms as a

consequence (Long *et. al.*, 1972; Reulen *et. al.*, 1972; Guseso & Jellinger, 1975; Barkhof *et. al.*, 1991). While in EAE such actions may account for the improvement in neurological deficit seen, they do not directly explain the restoration of BBB function. The actions of DEX which may indirectly influence neuroendothelial permeability will be considered first, with subsequent discussion of steroid effects directly at the BBB.

Glucocorticoid suppression of the T cell response is an important feature in the prevention of cell-mediated disease such as EAE (Serrano *et. al.*, 1993). However, while immunosuppression of lymphocyte activation is a major component of prophylactic treatment (Desai & Barten, 1989; Bolton & Flower, 1989), modulation of cytokine and vasoactive compound production will be of greater significance during therapy. The variety of prophylactic regimes for DEX, explored by Bolton & Flower (1989), indicated that the drug was most effective when administered from D7-11PI and not D0-4PI. Therefore, an important role in controlling inflammatory and vasoactive mediator synthesis is suggested for DEX. Inhibition of cytokine transcription by steroids has been demonstrated for IL 1, 2, 3 and 6, TNF and IFNγ (Arya *et. al.* ,1984; Reed *et. al.* ,1986; Culpepper *et. al.*, 1985; Helfgott *et. al.*, 1987; Lew *et. al.*, 1988; Lee *et. al.*, 1988; Waage & Bakke, 1988). Immunocytochemical techniques have demonstrated elevated levels of TNFα, IL-1, IL-2, IL-3 and IFNγ in CNS inflammatory sites during chronic-relapsing EAE (Baker *et. al.*, 1991). Furthermore, administration of IL-1 to EAE-sensitised Lewis rats was demonstrated to exacerbate both the severity and duration of disease (Jacobs *et. al.*, 1991). Similarly, analysis of MS brain lesions has identified increased expression of IL-1, IL-2 (Hofman *et. al.*, 1986) and TNF-α (Cannella & Raine, 1995). The monokines IL-1 and TNF are of particular interest as both have demonstrated vascular permeability inducing properties on endothelial cell lines (Brett *et. al.*, 1989; Royall *et. al.* ,1989). Furthermore, the elevated expression of lymphocyte adhesion molecules in the CNS of EAE animals (Wilcox *et. al.*, 1990; Barten & Ruddle, 1994;

O'Neill *et. al.*, 1991) can be demonstrated by addition of IL-1, TNF and IFNγ to endothelial cells *in vitro* (Dustin *et. al.*, 1986; Pober *et. al.*, 1986; Hughes *et. al.*, 1988; Carlos *et. al.*, 1990). Moreover, in a study of MS patients a correlation between BBB damage and circulating levels of intercellular adhesion molecule-1 and TNFα were found (Sharief *et. al.*, 1993). A restriction of inflammatory cytokine production by glucocorticoids could therefore limit both barrier breakdown and lymphocyte trafficking.

The potent vasoactive nitrogen intermediate nitric oxide (NO) appears to increase blood flow and plasma exudation at inflammatory sites. Indeed, elevated levels of nitrite have been detected during the onset and progression of EAE (Bolton *et. al.*, 1994; Scott *et. al.*, 1994). Moreover, administration of NO-releasing compounds has been demonstrated to alter BBB permeability in the normal rat brain (Shukla *et. al.*, 1996). Macrophages produce large quantities of inducible NO synthase when activated and following recruitment during EAE into perivascular inflammatory sites, are ideally located to influence BBB function through excess NO production. Furthermore, the macrophage inducible NO synthase is potently inhibited by steroids, indicating a potential action for DEX in maintaining barrier function during EAE (Radomski *et. al.*, 1990). In addition to the production of NO by inflammatory cells, the neuroendothelium also has the potential to release the mediator in response to cytokine stimulation (Durieu-Trautmann *et. al.*, 1993). Indeed, glucocorticoids have also been shown to reduce NO production and suppress associated cytotoxic effects when added to endothelial cell cultures (Radomski *et. al.*, 1990; Palmer *et. al.*, 1992).

Control of vasoactive amine release by mast cells is another putative site for steroid suppression of neurovascular dysfunction during EAE. Indeed, glucocorticoid administration has been demonstrated to inhibit histamine release from rodent mast cells (Fahey *et. al.*, 1981; Daëron *et. al.*, 1982). Orr & Stanley (1989) demonstrated elevation of histamine concentration in the CNS tissues of EAE-sensitised Lewis rats

at D11 and D13PI. Furthermore, the percentage of degranulated mast cells has been shown to be significantly increased in EAE brains beginning concomitant with the onset of symptoms at D10PI and peaking at D16PI (Bø *et. al.*, 1991). In non-disease models elevated endogenous histamine has also been implicated in the loss of BBB integrity following heat stress, where pretreatment with the histamine H_2 receptor antagonist cimetidine inhibited neurovascular perturbation (Sharma *et. al.*, 1992). In addition, exogenous histamine administered by the carotid artery was found to increase pinocytotic vesicle activity and albumin extravasation at brain capillary sites, through H_2 receptor interaction (Dux & Joó, 1982), findings typical of EAE BBB pathology (Claudio et. al., 1989; Leibowitz & Kennedy, 1972).

A potential BBB permeabilising action of histamine or indeed cytokine release is the induction of arachidonic acid metabolism at the neuroendothelium through increasing phospholipase A_2 activity (Blackwell *et. al.*, 1978). Ohnishi *et. al.* (1992) clearly demonstrated that arachidonic acid could initiate neuro-endothelial dysfunction in normal rats. Furthermore, the study indicated a role for DEX in the control of barrier permeability through suppression of the *de novo* protein synthesis of vasoactive metabolites. Endogenous steroid levels are normally sufficient to moderate prostaglandin synthesis as adrenalectomy causes increased levels of arachidonic acid metabolites in carrageenin pleurisy (Flower *et. al.*, 1986). Elevated concentrations of permeability inducing leukotrienes and prostaglandins have been reported during disease onset in acute EAE and in the CSF of MS patients (Bolton *et. al.*, 1984a,b,c; Neu *et. al.*, 1988). Glucocorticoid suppression of phospholipase A_2 is well documented (Blackwell *et. al.*, 1978; Russo-Marie *et. al.*, 1979; Hirata *et. al.*, 1980) and control of EAE with inhibitors of arachidonic acid metabolism is widely reported (Prosiegel *et. al.*, 1989; Fretland *et. al.*, 1991). However, Weber *et. al.* (1991) found that the cyclooxygenase inhibitor piroxicam was only effective in EAE prophylactically, with therapeutic administration being highly detrimental. The late blocking of the cyclooxygenase arm of arachidonic acid metabolism may therefore be

ineffective. Indeed, prostaglandins PGE and $PGF_{2\alpha}$ are reduced in the CSF of MS patients during a relapse (Bolton *et. al.*, 1984c). Moreover, if eicosanoid production varies during the course of autoimmune disease it may indicate why the ability of DEX to control neurovascular permeability also alters in acute and recovery stages.

The neurovascular protective actions suggested for glucocorticoids above, may be linked to immune cell actions. However, the neurovasculature has the ability to synthesise both NO and eicosanoids independent of an immunological stimulus (Joó, 1985; Radomski *et. al.*, 1990)) and histamine damage of BBB function has been recorded in a non-immune model (Sharma *et. al.*, 1992).

Whether induced by immune-derived mediators or vasoactive compounds, the resultant abnormal mechanisms leading to neurovascular disruption must focus at the level of the BBB. Analysis of abnormal barrier activity in non-immune models has provided a candidate pathway which may be controlled by glucocorticoid administration. Studies in hyperosmolar and cold injury induced models of BBB breakdown report increased activity of the enzyme ornithine decarboxylase (ODC) and elevated polyamine(PA) end products, concomitant with CNS microvascular permeability elevation (Koenig *et. al.*, 1989a; Koenig *et. al.*, 1989b). Furthermore, increased PA levels have been detected in the brains of EAE animals displaying neurological deficits (Bolton *et. al.*, 1994; and unpublished observations). Administration of DEX at the time of cold injury inhibited both enhanced ODC activity and PA concentration concurrent with an attenuation of BBB dysfunction (Koenig *et. al.*, 1989b). The mechanism of action proposed for DEX by Koenig and co-workers was through the suppression of arachidonic acid release which precedes ODC increases in the cold-injured brain. However, the molecular mechanisms underlying the two events remain to be clarified. Interestingly, while large steroid doses successfully abolish abnormal levels of arachidonic acid (Politi *et. al.*, 1985; De Kloet *et. al.*, 1983), low concentrations reportedly enhance ODC levels in normal or adrenalectomised rats (Ikeno *et. al.*, 1978) and fail to inhibit acute release of

arachidonic acid in cryoinjured brain (Pappius & Wolff, 1983). The effect noted with the lower doses of administered glucocorticoid may be indicative of normal homeostatic control of ODC activity by endogenous steroids (Meyer, 1985). Indeed, ODC control appears to closely correlate with steroid affinity for brain glucocorticoid receptors (Cousin *et. al.*, 1982).

The present work has shown that therapeutic administration of DEX dose-dependently suppresses BBB opening during the acute phase of EAE and restricts BBB permeability during recovery. The action of the steroid primarily involves binding to the type II glucocorticoid receptor. While the mechanism of control of neurovascular permeability in EAE by therapeutically administered DEX remains to be defined, the ODC cascade has been recognised as a key neuroendothelial target.

6.
Modulation of BBB Dysfunction by Immunosuppressive Agents Cyclosporin A and FK506

6.1 Results

6.1.1 Therapeutic Administration of CSA in the Acute Phase of Disease

Therapeutically administered CSA has been reported to reduce the severity and incidence of symptoms and decrease the number of CNS inflammatory lesions that develop during EAE (Bolton *et. al.*, 1982; Rumjanek *et. al.*, 1984b). Whether the drug works in part through actions at the BBB has not been investigated and no study of the modulatory effect of therapeutic CSA on neurovascular permeability has been reported.

Experiments were conducted to evaluate the efficacy of a range of CSA doses to control the abnormal neurovasculature function during an acute episode of EAE. Doses ranged from 25 to 75mg/kg body weight and were administered in a short-term therapeutic regime starting on the day of weight loss.

The results show CSA dose-dependently restricts the irregular BBB opening associated with acute EAE (Figure 31a-c). Low doses of 25 and 35mg/kg body weight CSA did not alter neurovascular permeability. However, the 35 mg/kg dose of CSA did significantly reduce the MNS for the treatment group. Increasing the concentration of immunosuppressant to 50mg/kg body weight was sufficient to lower the EVBE value in the C and to significantly limit neurovascular dysfunction in the MP and CSp tissues ($p < 0.01$ and $p < 0.001$ respectively). A marked reduction in MNS was also observed (Table 7). A further increase in dose to 75mg/kg CSA achieved significant inhibition of BBB breakdown in all tissues including the C ($p < 0.001$), but the improvement from the 50mg/kg dose was unremarkable and barrier permeability remained significantly different to normal control values (Figure 31). Analysis of CSA log response data showed a significant linear regression in all tissues (C, $p = 0.002$, 21% fit; MP, $p = 0.0$, 46% fit; CSp, $p = 0.0$, 62% fit).

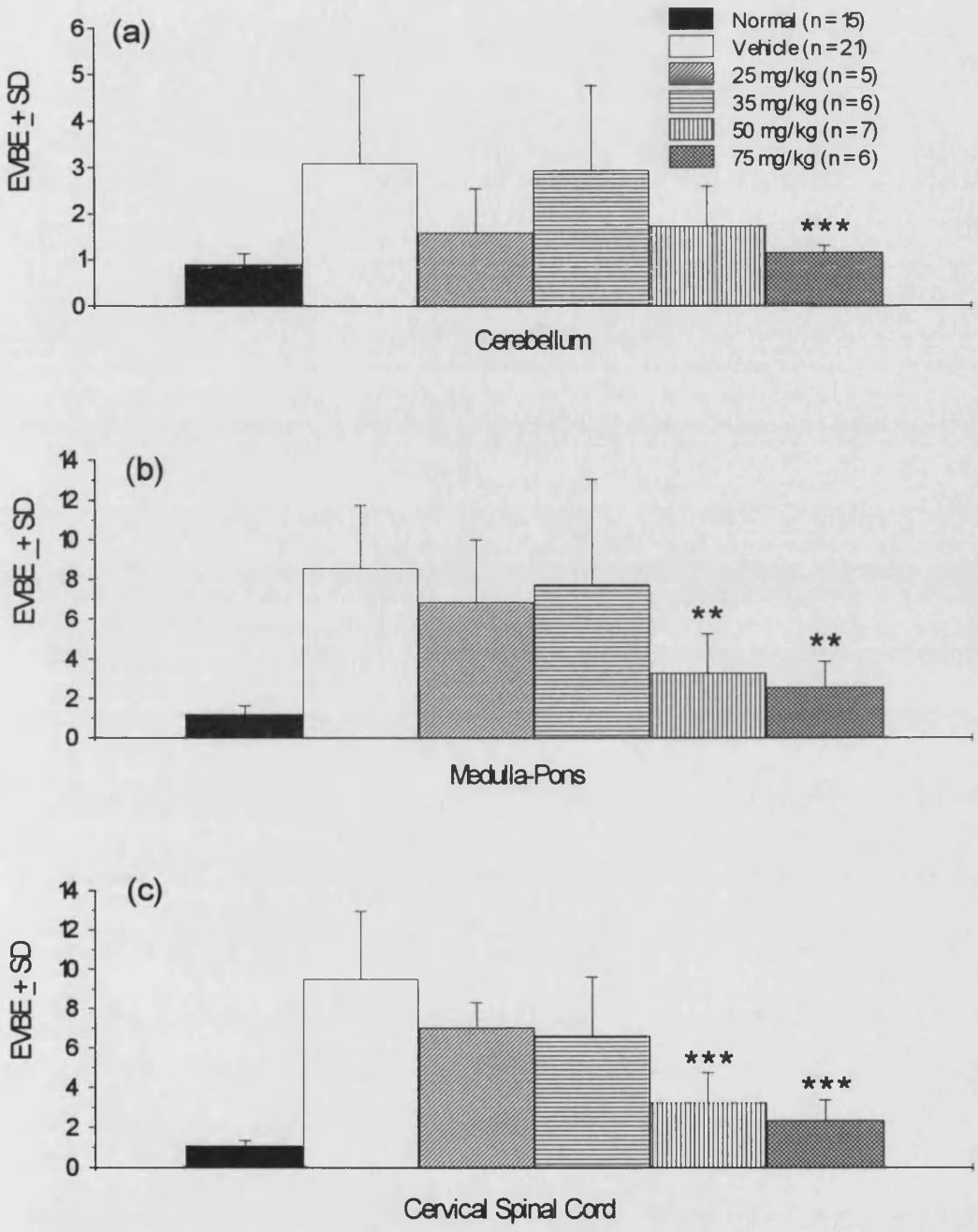


Figure 31: Inhibition of BBB breakdown by CSA Administered During the Acute Phase of EAE. CSA was administered at doses of 25 (n = 5), 35 (n = 6), 50 (n = 7) and 75 (n = 6) mg/kg body weight, alongside normal (n = 15) and vehicle (n = 21) controls. Dosing was once daily for two days starting on the day of weight loss. The graphs show BBB dysfunction in the **(a)** cerebellum, **(b)** medulla-pons and **(c)** cervical spinal cord. Analysis of key doses was by Mann-Whitney U test with Bonferroni correction where required; ** $p < 0.01$ and *** $p < 0.001$ compared to vehicle control. The 75mg/kg dose was found to be significantly different from normal in all CNS areas, C $p = 0.036$, MP $p = 0.028$ and CSp $p = 0.005$; Mann-Whitney U. Analysis of the log data showed a significant linear regression in all tissues (C, $p = 0.002$, 21% fit; MP, $p = 0.0$, 46% fit; CSp, $p = 0.0$, 62% fit).

The effect of CSA on lesion formation in the CSp was analysed at two concentrations. Low dose treatment with 25mg/kg CSA did not affect the accumulation of perivascular infiltrates. However, high dose CSA (50mg/kg) significantly reduced lesion formation in the CSp compared to the untreated EAE controls ($p < 0.05$). Complete suppression of cellular inflammation was not achieved.

Table 7: Summary of Neurological Status, Histological Scores and Plasma Corticosterone Levels following CSA Administration in the Acute Phase of EAE

	n	MNS ± SD	n	CSp Lesion no. ± SD	n	[Corticosterone] ng/ml ± SD
Normal	-	-	-	-	5	76.8 ± 18.9
D13 PI EAE	8	1.63 ± 1.75	8	117.0 ± 61.0	-	-
Vehicle	21	1.7 ± 1.0	-	-	5	179.2 ± 64.0
25 mg/kg CSA	5	1.2 ± 1.6	5	148.4 ± 56.1	-	-
35 mg/kg CSA	6	0.2 ± 0.4*	-	-	-	-
50 mg/kg CSA	7	0.2 ± 0.4*	5	50.8 ± 20.1#	7	110.9 ± 48.2
75 mg/kg CSA	6	0*	-	-	-	-

Experimental groups match those described in Figure 31. CSA was dosed once daily for two days starting on the day of weight loss and concluding on the day prior to sampling. MNS were recorded on the day of sampling and analysed by Dunnett's test following ANOVA; * $p < 0.01$ compared to vehicle control. Histology values represent the number of lesions seen per whole section of tissue; # $p < 0.05$, T-test compared to control. No significant difference between drug and vehicle groups was demonstrated for the corticosterone data; Mann-Whitney U.

In order to exclude the possibility of CSA administration modifying aspects of disease, including barrier function, through elevation of endogenous steroid production, plasma samples from experimental groups were analysed to determine corticosterone levels. Results showed no significant difference between the amounts

of corticosterone measured in 50mg/kg body weight drug treatment and vehicle control groups (Table 7).

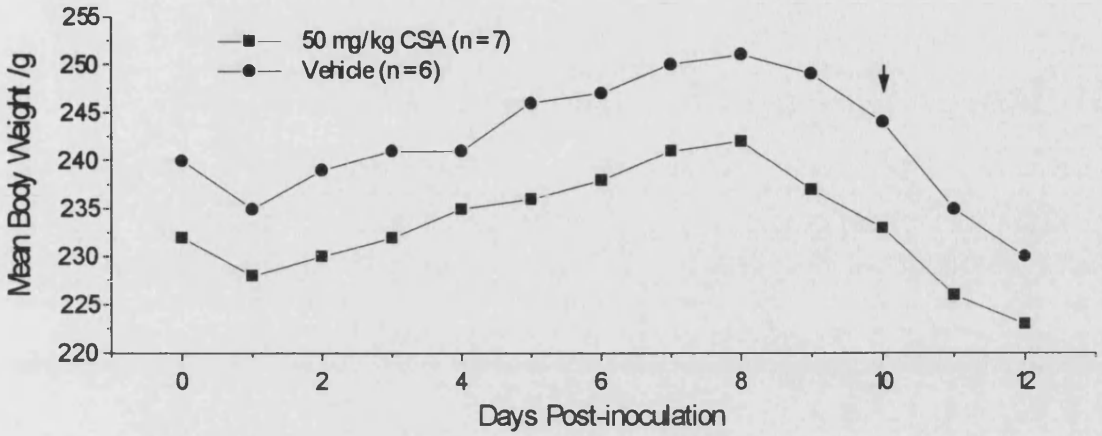
An elevated plasma creatinine concentration is a characteristic feature of renal dysfunction, a potential side effect of CSA treatment (Schulman *et. al.*, 1981; Mihattsch *et. al.*, 1988). Therefore, to assess the tolerance of the rats to the short-term therapeutic regime the plasma creatinine level and body weight profile of treated animals were analysed. A study of plasma from EAE-sensitised animals dosed with 35 and 75mg/kg CSA did not show significant elevation of creatinine levels above normal and vehicle control values (Figure 32). Furthermore, the data obtained was within the normal concentration range for plasma creatinine of 0.4-3.7mg/dL for rats (Clinical Biochemistry of Domestic Animals, 1989). Figure 32 shows that the body weight profile of animals receiving 50mg/kg body weight CSA compared closely to the vehicle dosed control group. Further analysis of the change in body weight over the period of dosing found no difference in weight loss between any of the treatment groups under study. Therefore, CSA doses administered therapeutically appear to have been well tolerated.

6.1.2 Administration of CSA during the Early Recovery Phase of Disease

The sensitivity of BBB function to control by DEX appeared to vary between acute and early recovery phases of EAE. Therefore, a study was undertaken to determine whether the efficacy of CSA action at the neurovasculature also altered between acute and recovery timepoints. Administration of CSA at 25 and 50mg/kg began after the loss of neurological deficits using groups of EAE-sensitised animals which had displayed severe signs of disease during the acute phase.

EVBE calculations of barrier permeability to protein show a marked improvement in all tissues with the administration of 25mg/kg CSA (Figure 33). Low dose CSA had previously shown no neurovascular-protective effect during the acute

(a)



(b)	n	% Change in Body Weight[a]	n	[Creatinine] mg/dL-1
Normal	-	-	6	0.335 ± 0.074
Vehicle	21[b]	9.5 ± 3.0	10[c]	0.341 ± 0.114
25 mg/kg CSA	5	7.5 ± 3.8	-	-
35 mg/kg CSA	6	8.0 ± 2.8	5	0.388 ± 0.062
50 mg/kg CSA	7	8.7 ± 3.3	-	-
75 mg/kg CSA	6	5.5 ± 2.6	6	0.384 ± 0.128

[a] : Percentage change in body weight between peak weight prior to disease onset and the day of sampling.
[b] : Combined vehicle results from four experiments
[c] : Combined vehicle results from two experiments

Figure 32: Analysis of Body Weight Changes and Plasma Creatinine Concentration in CSA Treated EAE-sensitised Animals. (a) Weight profiles of rats treated with 50mg/kg CSA (—■—, n = 7) and vehicle-dosed controls (—●—, n = 6). Treatment was initiated at weight loss (arrow) and continued for two days. **(b)** Percentage body weight change over treatment period for all CSA doses plus plasma creatinine concentrations from control and 35 and 75mg/kg dosed animals. ANOVA of both % weight change and plasma creatinine concentration found no differences between groups.

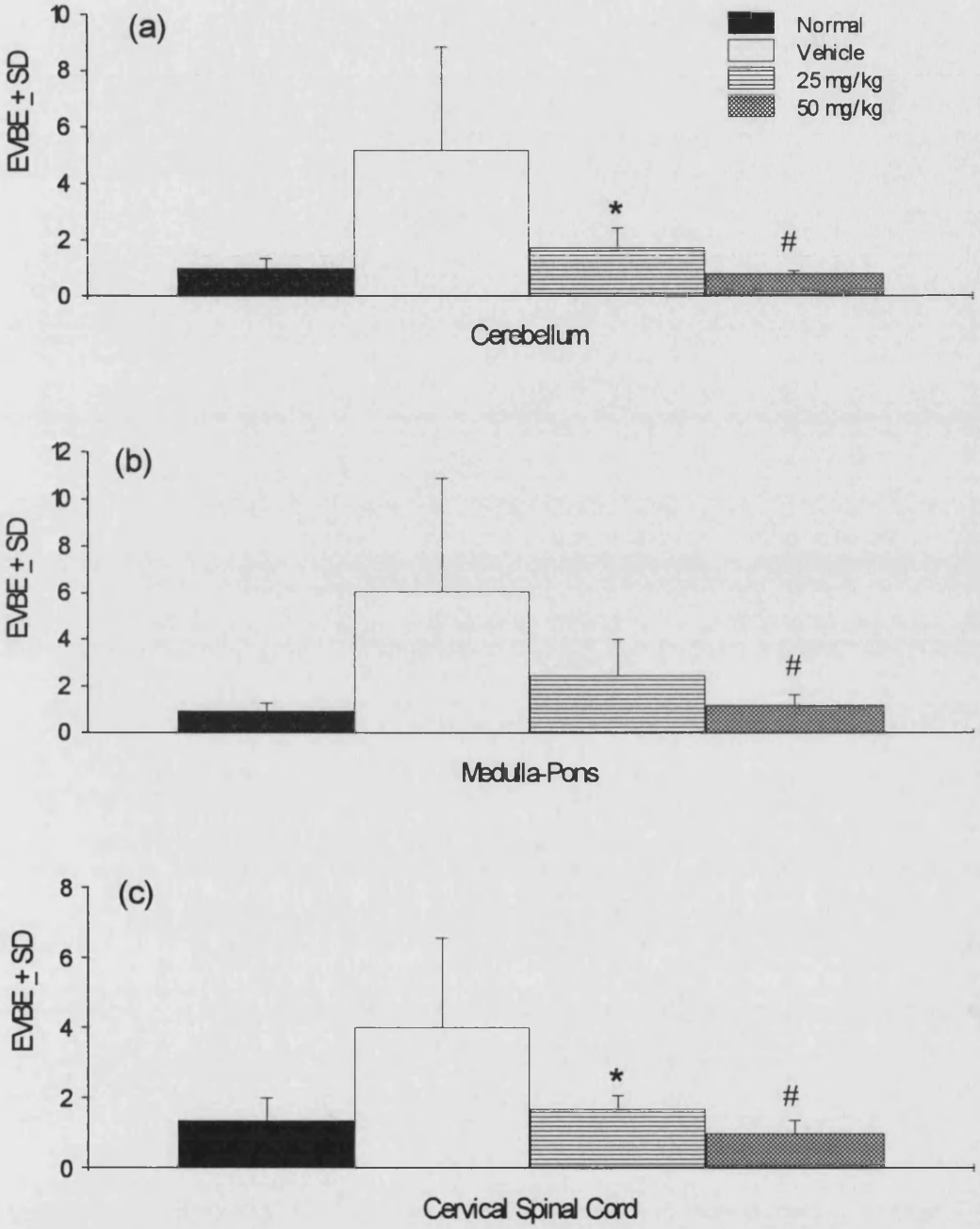


Figure 33: The Effect of CSA on Abnormal BBB Function during Early Recovery in Acute EAE. CSA was administered at 25 or 50mg/kg body weight for two days beginning after the loss of disease symptoms and two consecutive days of weight gain. Animals had shown a minimum neurological score of 3 during the acute phase. The histograms show pooled data from two experiments for **(a)** cerebellum, **(b)** medulla-pons and **(c)** cervical spinal cord tissues, where: normals (black column, n = 5), vehicle controls (open column, n = 8), 25mg/kg CSA (horizontal hatch column, n = 4) and 50mg/kg CSA (cross hatch column, n = 5). Mann-Whitney U analysis of data: * $p < 0.05$ compared to vehicle and # no significant difference to normal. Analysis of the log data showed a significant linear regression in all tissues (C, p = 0.0, 68% fit; MP, p = 0.0, 62% fit; CSp, p = 0.0, 66% fit).

phase of disease but significantly lowered BBB permeability in C and CSp tissues ($p < 0.05$) during recovery. Administration of CSA at 50mg/kg did not normalise barrier permeability in acute disease, but reduced EVBE values to normal levels when given during the early recovery phase (C, $p = 0.403$; MP, $p = 0.296$; CSp, $p = 0.295$). The linear regression of log response against drug concentration was significant with a >60% fit in all tissues. An increase in neurovascular sensitivity to administered CSA is therefore indicated during early recovery in EAE.

6.1.3 Therapeutic Administration of FK506 in the Acute Phase of Disease

The novel macrolide FK506 has a suppressive action in both active and adoptively transferred EAE (Deguchi *et. al.*, 1991; Bolton, 1992). In addition, FK506 is approximately 100-fold more effective than CSA at inhibiting passive transfer of neuroantigen-induced disease (Bolton, 1992). Therefore, it was of interest to discover whether FK506 could inhibit abnormal neurovascular function to an equal or greater degree than CSA.

EVBE values show no reduction in abnormal barrier extravasation of protein for the 5mg/kg FK506 oral preparation (Figure 34a). Indeed, a significant increase above the mean EVBE value for the vehicle group was found in the CSp tissue ($p < 0.05$). Neurological symptoms were not suppressed.

An increase to 10mg/kg body weight FK506 administered i.p., dramatically improved the ability of FK506 to restrict BBB opening in the acute phase of EAE (Figure 34b). The dose significantly reduced barrier abnormalities in the MP and CSp tissues ($p < 0.05$) maintaining the mean EVBE within normal limits. The experiment was unusual in that no elevation in BBB permeability was noted in the C. However, the neurovascular opening in MP and CSp tissues attained levels of dysfunction typical for acute EAE and a significant drug effect could be demonstrated for these areas. The study also produced an abnormally low MNS in the vehicle group

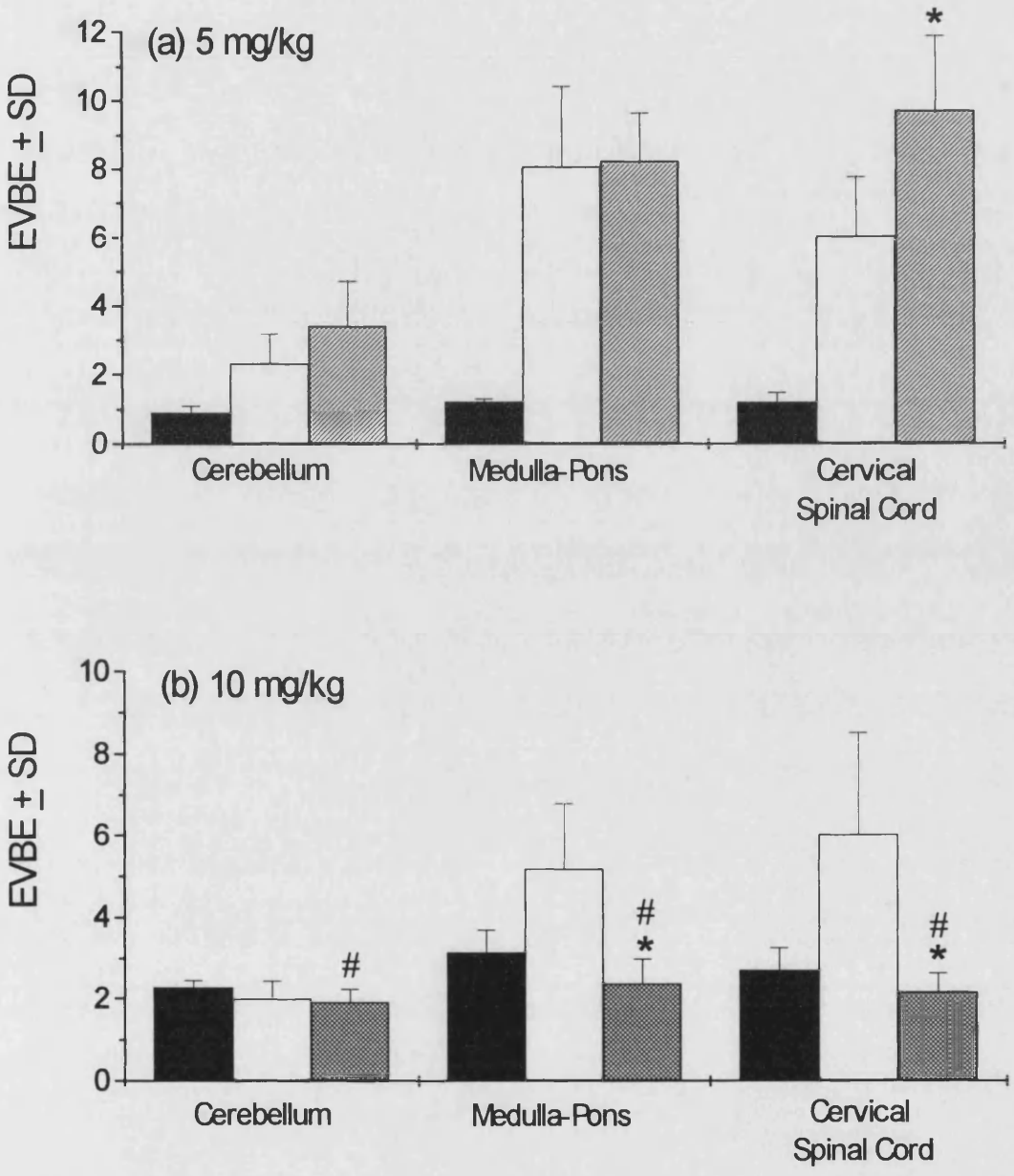


Figure 34: Inhibition of BBB breakdown by FK506 Administered During the Acute Phase of EAE. The effect of FK506 on BBB permeability in C, MP and CSp tissues was studied over the course of two experiments. Graph **(a)** FK506 administered at 5mg/kg as an oral suspension in PBS (diagonal hatch column, n = 6) and compared to normal (black column, n = 4) and vehicle (open column, n = 7) controls. Graph **(b)** FK506 administered at 10mg/kg i.p. dissolved in PBS with 10% ethanol and 2% Tween (cross hatch column, n = 7) and compared to normal (black column, n = 6) and vehicle (open column, n = 5) controls. Dosing was once daily for two days starting at weight loss. Statistical analysis showed differences of * $p < 0.05$ between drug and vehicle groups and # no significant difference between drug and normal groups; Mann-Whitney U test.

preventing a reduction in symptoms from being seen in the drug treated animals (Table 8). However, histological assessment of CNS lesions did show a marked inhibition of inflammatory infiltrates following FK506 administration (Table 8). Furthermore, plasma corticosterone was not elevated in the drug-treated group, eliminating the possilbility of FK506 administration suppressing EAE development through upregulation of endogenous steroids (Table 8).

Analysis of the body weight profile of EAE-sensitised animals receiving 10mg/kg FK506 did not show extensive weight loss during treatment compared to the vehicle-treated EAE rats (Figure 35). The maximum dose of FK506 employed therefore seemed well tolerated.

Table 8: Summary of Neurological Status, Histological Scores and Plasma Corticosterone Levels following FK506 Administration in the Acute Phase of EAE

	n	MNS ± SD[c]	n	CSp Lesion no. ± SD[d]	n	[Corticosterone] ng/ml ± SD[e]
Normal	-	-	-	-	3	63.0 ± 41.7
D13PI EAE	8	1.6 ± 1.8	8	117.0 ± 61.0	-	-
Vehicle[a]	7	1.1 ± 1.5	-	-	-	-
5 mg/kg FK[a]	6	2.1 ± 1.9	-	-	-	-
Vehicle[b]	5	0.5 ± 0.5	-	-	3	94.7 ± 34.3
10 mg/kg FK[b]	7	0.1 ± 0.2	2	2.5 ± 2.1	5	59.2 ± 13.8

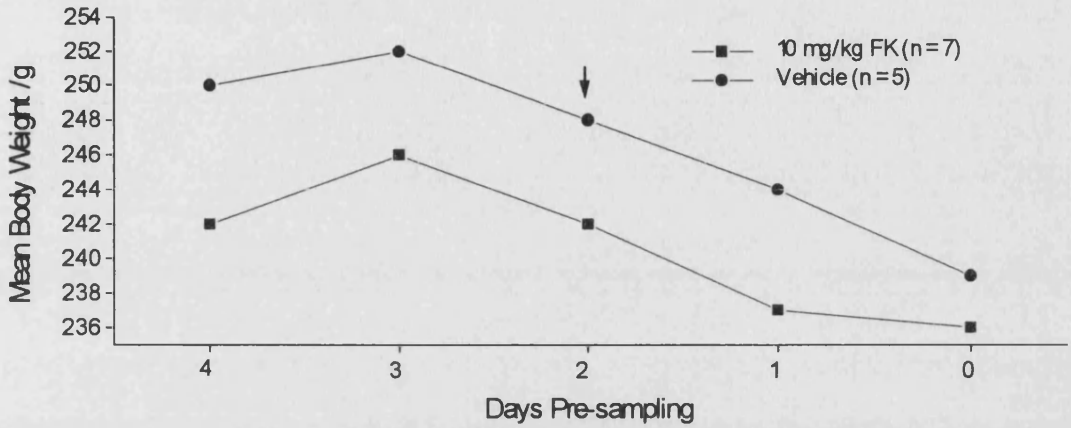
[a, b] : Denote groups from same experiment

[c] : MNS observed on the day of sampling. No significant difference between vehicle and drug groups was shown by Mann-Whitney U assessment.

[d] : Lesion numbers reflect the number of inflammatory cuffs seen in a whole CSp section.

[e] : Plasma corticosterone levels: No significant differences between groups by ANOVA determination.

(a)



(b)

	Vehicle[a]	5 mg/kg FK[a]	Vehicle[b]	10 mg/kg FK[b]
% Weight Change[c]	9.3	10.0	5.1	4.9
SD	3.0	1.9	2.4	1.7
n	7	6	5	7

[a, b] : Denote groups from same experiment

[c] : % weight change between pre-disease weight and that measured on the day of sampling

Figure 35: Analysis of Body Weight Changes in FK506 Treated EAE-sensitised Animals. (a) Weight profiles of rats treated with 10mg/kg FK506 (—■—, n = 7) and vehicle-dosed controls (—●—, n = 5). Treatment was initiated on the day of weight loss (arrow) and continued for two days. **(b)** Percentage body weight change during treatment. No differences between vehicle and drug groups were shown; T-test.

6.1.4 Administration of FK506 during the Early Recovery Phase of Disease

The efficacy of 10mg/kg FK506 was investigated in the recovery phase of EAE where the neurovascular abnormalities are continued in the absence of disease symptoms. The animals studied had displayed severe neurological symptoms during the acute phase of disease.

Figure 36 describes BBB dysfunction in the experimental groups. Dosing with FK506 at 10mg/kg demonstrated a correction of barrier function in C and CSp tissues equal to that seen during treatment in the acute phase ($p < 0.05$). Neither the MP or the CSp were determined to be significantly different from normal values. However, a single value greater than 1.2 EVBE prevented significant inhibition of neurovascular permeability from being attained in the MP when compared to vehicle control values. Therefore, the results suggest a similar correction of neurovascular abnormalities by FK506 at10mg/kg in both the acute and recovery stages of disease.

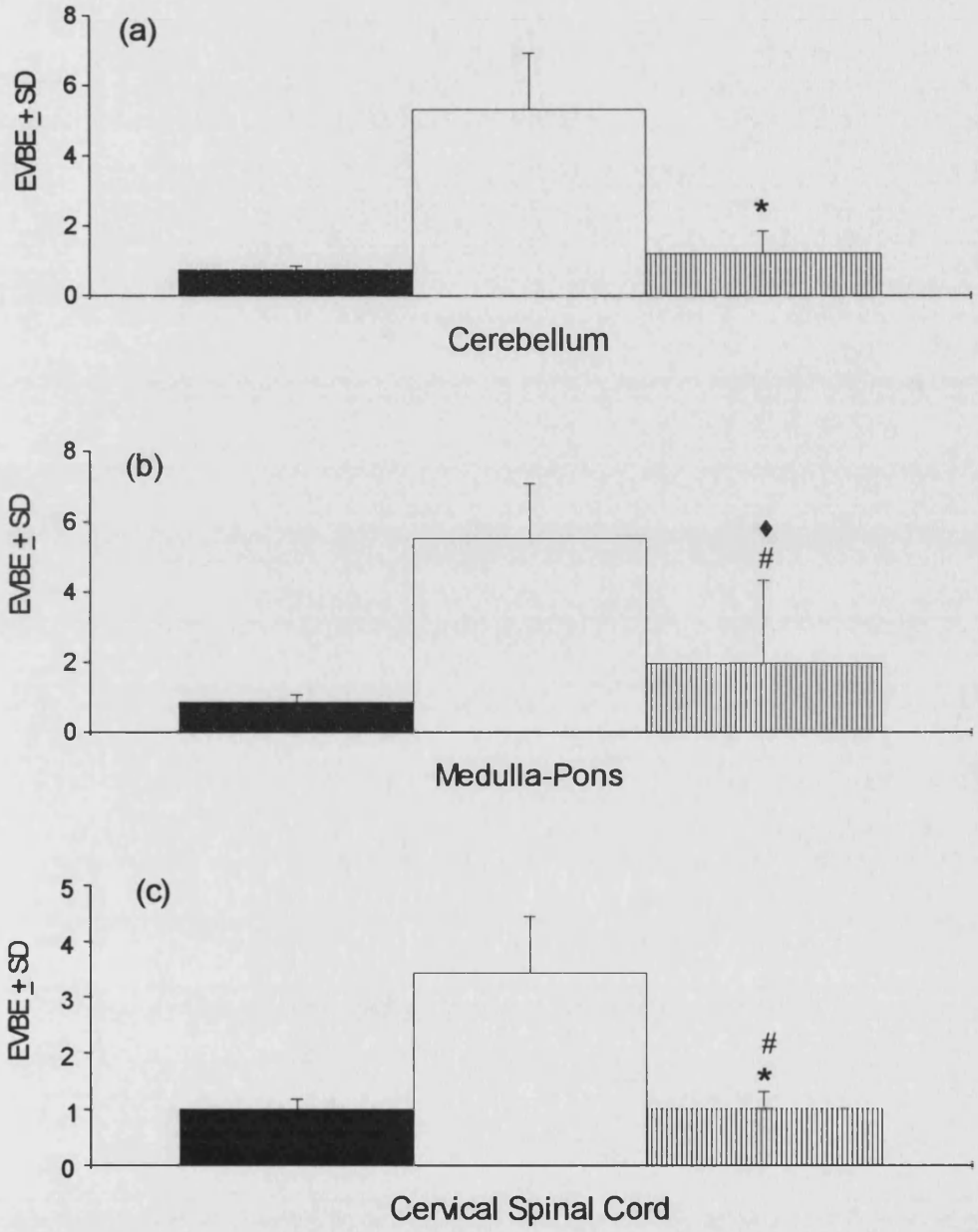


Figure 36: The Effect of FK506 (10mg/kg body weight) on Abnormal BBB Function during Early Recovery in Acute EAE. FK506 was administered at 10mg/kg body weight for two days beginning after the loss of symptoms and two consecutive days of weight gain. Animals had shown a minimum neurological score of 2.5 during the acute phase. The graphs show the normal (black column, n = 4), vehicle (open column, n = 5), and 10mg/kg FK506 (vertical hatch column, n = 6) results in **(a)** cerebellum, **(b)** medulla-pons and **(c)** cervical spinal cord tissues. Mann-Whitney U analysis of data showed, * $p < 0.05$ compared to vehicle and # no significant difference to normal. ♦ only one value exceeded 1.2 EVBE.

6.2 Discussion

CSA administered by short-term therapy demonstrated a significant suppression of BBB abnormalities, neurological symptoms and histologically recognised lesions, during the acute phase of EAE. Interestingly, administration of the drug during the recovery stage demonstrated a greater ability to normalise BBB function.

The dose-dependent reduction of neurological and histological signs of EAE reconfirms the observations of therapeutic studies previously reported (Bolton *et. al.*, 1982; Rumjanek *et. al.*, 1984b). Furthermore, reports of CSA use in other experimental inflammatory conditions also demonstrate therapeutic control at comparable doses (Borel *et. al.*, 1976; Pozo *et. al.*, 1992). Significant suppression of EAE has been reported at lower concentrations of CSA, but only in prophylactic dosing regimes (Hinrichs *et. al.*, 1983; Reiber & Suckling, 1986).

An isolated study by Reiber & Suckling (1986) had previously examined BBB function following CSA administration in EAE. The investigation found that CSA treatment, from one day pre-sensitisation, suppressed disease development and inhibited the increases in CSF protein concentration, which normally indicates barrier dysfunction. In the present study CSA has been shown to reduce the opening of the BBB when administered for a short time period therapeutically. Moreover, while the maximal effect of CSA in the acute phase does not achieve complete correction, the immunosuppressant can normalise protein extravasation at D23PI. Indeed, the observed difference between EAE timepoints reinforces the observations made with DEX treatment, indicating that the neurovascular response to drug administration alters between acute and recovery phases.

FK506 administered therapeutically, significantly restricted BBB opening, neurological deficits and histological lesions in the Lewis rat model of acute EAE.

The effect of FK506 on the pathological breakdown of neurovascular function has not previously been shown. This study has demonstrated that the macrolide can both prevent BBB perturbation in the acute phase and restore barrier function at D23PI after only a short dosing regime. Previous work in EAE has shown that FK506 can successfully suppress onset of disease when administered at low doses from D0PI (Inamura *et. al.*, 1988; Deguchi *et. al.*, 1991). However, Deguchi *et. al.* (1991) reported irritability and death in animals treated with 10mg/Kg of FK506 for 12 consecutive days. Intolerance to FK506 was not noted at this concentration during the current study when used in a short dosing regime. Furthermore, a similar dose was well tolerated in collagen-induced arthritis when given as a single injection (Arita *et. al.*, 1989, 1990). The adverse reaction to FK506 at the dose reported by Deguchi *et. al.* (1991) maybe as a result of an accumulative effect. Accumulation of drug may explain why a lower dose of FK506 produced a response prophylactically in EAE, but was not successful in the short therapeutic regime reported here. In contrast to CSA and DEX, the efficacy of FK506 correction of BBB permeability was not found to alter between acute and recovery phases. Therefore, FK506 may target an aspect of neurovascular abnormality of equal importance in both BBB breakdown and continued vascular dysfunction. A full dose-response study in both acute and recovery stages of EAE could not be undertaken due to restricted availability of the compound.

The application of immunosuppressants CSA and FK506 to inflammatory and immune-mediated conditions is continually widening (reviews: Hess, 1993; Thomson *et. al.*, 1993). However, the mechanisms by which the compounds mediate improvement in disease are not clearly defined.

Pharmacological control of initial BBB breakdown and the sustained dysfunction of the microvasculature, may be exerted at various stages following inoculation for EAE. A primary target of CSA and FK506 in EAE is the inhibition of T cell activation and proliferation. While CSA and FK506 have distinct cellular

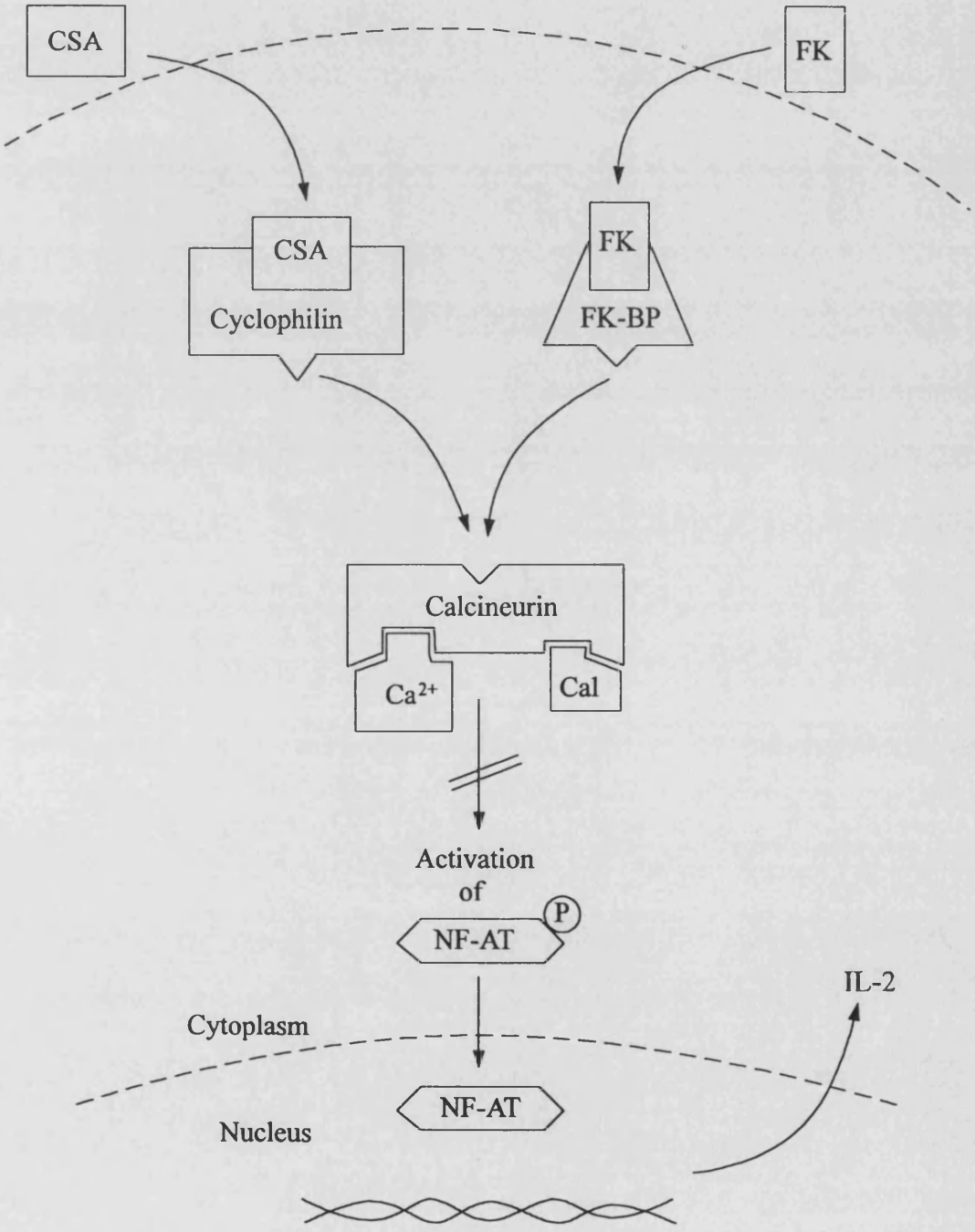
binding proteins, cyclophilins and FK-binding proteins respectively, the two drugs exhibit essentially identical effects on T cell inactivation. Inhibition of T cell proliferation by the immunosuppressants is essentially mediated by a down-regulation of IL-2 synthesis, as summarised in Figure 37.

The successful prophylactic suppression of CSA and FK506 in acute EAE and the inhibition of adoptively transferred EAE and antigen arthritis by T cell culture with immunosuppressants, may be attributed to the restriction of lymphocyte activation by the drugs (Hinrichs *et. al.* ,1983; Inamura *et. al.*, 1988; Bolton, 1992; Griffiths *et. al.* ,1992). However, therapeutic administration of CSA and FK506 occurs after lymphocyte priming and the early contact between activated surveillance cells and the CNS environment (Daniel *et. al.*, 1981; Traugott, 1989), suggesting that the compounds therapeutically suppress EAE by actions other than the inhibition of T cell activation.

Suppression of vasoactive mediator synthesis by immune cells could result in control of vascular permeability, particularly if the release from perivascular inflammatory cells was restricted. Both CSA and FK506 inhibit a variety of immune cells from producing cytokines (Goldfeld *et. al.*, 1992; Lafferty *et. al.*, 1983), including IL-1, TNFα and IFNγ, which have been implicated in microvascular permeabilisation and upregulation of adhesion molecules on the endothelium (Royall *et. al.*, 1989; Brett *et. al.*, 1989; Dustin *et. al.*, 1986). Release of proinflammatory mediators histamine, prostaglandin D_2 and leukotriene C_4 by mast cells and basophils are also inhibited by CSA and FK506 (Marone *et. al.* ,1988; Cirillo *et. al.* ,1990). Furthermore, CSA-induced inhibition of prostacyclin production from endothelial cells has been demonstrated *in vitro* (Brown *et. al.*, 1990). However, the suppression of arachidonic acid metabolite synthesis would not appear to be a general effect but directed at specific eicosanoids. Prostaglandins, particularly of the E series, inhibit IL-2 synthesis, a known target for immunosuppressants (Hess, 1993) and reports by

Figure 37: Proposed mechanism of CSA and FK506 inhibition of IL-2 stimulated T-cell proliferation

CSA and FK506 bind to soluble intercellular isomerases, cyclophilins and FK-binding proteins respectively. The drug-isomerase complex associates with the Ca^{2+} and calmodulin (Cal)-dependent phosphatase, calcineurin, inhibiting enzyme activity. As a consequence dephosphorylation of substrate proteins, including the cytoplasmic component of 'nuclear factor of activated T-cells' (NF-AT) does not occur. Translocation of NF-AT to the nucleus is subsequently prevented and IL-2 transcription down-regulated. (Sources: Henderson *et.al.*, 1991;Liu, 1993; Woerly *et.al.*, 1994)



Whistler *et. al.* (1984) and Schultze *et. al.* (1984) demonstrate CSA enhancement of prostaglandin synthesis in the monocytes.

Access of immunosuppressants to the neuroendothelium and the CNS parenchyma is possible, but restricted to a greater extent than elsewhere in the body. The lipophilic nature of CSA allows drug uptake to the brain at the same rate as uptake into the kidney (Nooter *et. al.*, 1984), although CNS concentrations are reportedly below detection limits (Palestine *et. al.*, 1985). One explanation may be the affinity of the drug to P-glycoprotein, a transmembrane efflux transporter located on the luminal membrane of neuroendothelial cells, which actively prevents many lipophilic molecules from gaining access to the CNS (Sakata *et. al.*, 1994; Wang, 1995; Schinkel *et. al.*, 1995). However, removal of drugs by the pump may be slow as Begley *et. al.* (1990) report quantifiable accumulation of CSA within the neuroendothelium from where direct control over BBB events may be initiated. Macrolides possess a poor blood to brain penetration yet the greater potency of FK506 may explain why the drug can exert a neuroprotective effect at CNS sites (Kitamura *et. al.*, 1994). Indeed, rat brain has demonstrated very high levels of FK-binding protein with co-localisation of calcineurin, indicating a possible physiological link through which FK506 may exert an effect (Steiner *et. al.*, 1992).

The ability of FK506 and CSA to act on the vascular endothelium is demonstrated in a number of toxic side-effects linked to administration of the drugs following transplant including hypertension (Rego *et. al.*, 1991) and vasculitis (Thriru *et. al.*, 1987). However, drug actions detrimental in normal tissues may be of benefit under pathological conditions. Indeed, CSA-induced nephrotoxicity appears to result from a suppressed NO production, which can be overcome by addition of the NO substrate L-arginine (Gallego *et. al.*, 1993). Nitrite levels, an indirect measure of NO, have recently been shown to be elevated in brain homogenates from EAE-sensitised animals at the time of disease onset. (Bolton *et. al.* ,1994; Scott *et. al.*, 1994). Furthermore, as a potent vasodilator NO has been linked to perturbation of vascular

endothelium in other systems (Palmer *et. al.*, 1992; Schukla *et. al.*, 1996). While CSA and FK506 may affect endothelial NO production the drugs could also reduce the level of the vasodilator released by macrophages situated within inflammatory CNS lesions in EAE (Conde *et. al.*, 1995).

Decreased mitochondrial content is a recognised ultrastructural change in the neuroendothelial cells of EAE-sensitised animals (Claudio *et. al.*, 1989), which has also been observed in biopsy samples from chronic progressive MS recently (Claudio *et. al.*, 1995). Mitochondria are sensitive to oxidative damage which inactivates mitochondrial enzymes and disrupts mitochondrial calcium metabolism, contributing to cell damage (Packer & Murphy, 1995). Interestingly, CSA has the potential to protect mitochondria by blocking the induction of calcium efflux and thereby may prevent endothelial changes in EAE (Packer & Murphy, 1994, 1995; Schweizer & Richter, 1996).

In addition to the cyclophillin proteins, CSA may also bind other cytosolic proteins including ODC (Ryffel, 1993). The ODC cascade and endproduct PA have been implicated in the neurovascular dysfunction of non-disease models (Koenig *et. al.* ,1989b) and more recently in EAE (Bolton *et. al.*, 1994; Paul *et. al.*, submitted 1996). Therefore, CSA binding to ODC may reduce neurovascular perturbation by suppressing enzymic activity. Indeed, CSA has been observed to inhibit ODC activity (Fidelius *et. al.*, 1984), although a mechanism of action was not defined.

The mechanisms of action of therapeutically administered FK506 and CSA in EAE are uncertain although a number of actions of potential relevance to the control of BBB permeability have been suggested. A further area for investigation could include the analysis of the related immunosuppressants rapamycin and cyclosporin H on BBB function during EAE. Rapamycin also targets the cytosolic FK-binding proteins but demonstrates an alternative method of immunosuppression to FK506 and may indicate the extent of FK-binding protein involvement in the mechanisms controlling BBB improvement. Cyclosporin H is an analogue of CSA and possesses

no immunosuppressive actions. Therefore, restriction of BBB dysfunction during acute and recovery phases of EAE with this analogue would indicate a direct and non-immunosuppressive action of CSA on the neuroendothelium.

7.
Combined Administration of Cyclosporin A and Dexamethasone during the Acute Phase of EAE

7.1 Results

The contrasting efficacies of CSA and DEX during acute and early recovery stages of EAE suggest the compounds restore barrier permeability by different routes. Studies were therefore instigated to consider the effect of combined therapy in neuroantigen-induced disease.

A study was undertaken using DEX at a dose with negligable inhibitory effect on abnormal BBB function - 0.1mg/kg body weight - in combination with CSA doses of 25, 35 and 50mg/kg body weight, which range from ineffective to partially corrective concentrations. DEX plus 25mg/kg CSA was unable to improve the function of the BBB displaying a tissue profile similar to that of CSA alone and losing the minor effect in the CSp displayed by single DEX administration (Figure 38). Surprisingly, the combination of 0.1mg/kg DEX and 25mg/kg CSA was able to inhibit the development of symptoms unlike the individually administered drugs (Table 9).

Treatment with DEX (0.1mg/kg) and CSA at 35mg/kg body weight caused a dramatic improvement in neurovascular permeability ($p < 0.05$), which was determined to be within normal limits in all tissues (Figure 39). Complete normalisation of barrier function was not achieved by any CSA dose alone. Moreover, the % inhibition of BBB opening by the combined drugs was far greater in all CNS tissues than the predicted additive effect from single dose studies, indicating a synergistic effect of the two compounds at these concentrations (C: single 7%; combined 95%. MP: single 21%; combined 88%). In addition, neurological deficits were significantly reduced (Table 9).

A further increase of CSA to 50 mg/kg body weight in the combined dose maintained normal barrier function, reducing the group variation (SD) still further (Figure 40). A synergistic effect was again produced in the C and MP tissues (C: single 62%; combined 100+%. MP: single 82%; combined 100+%).

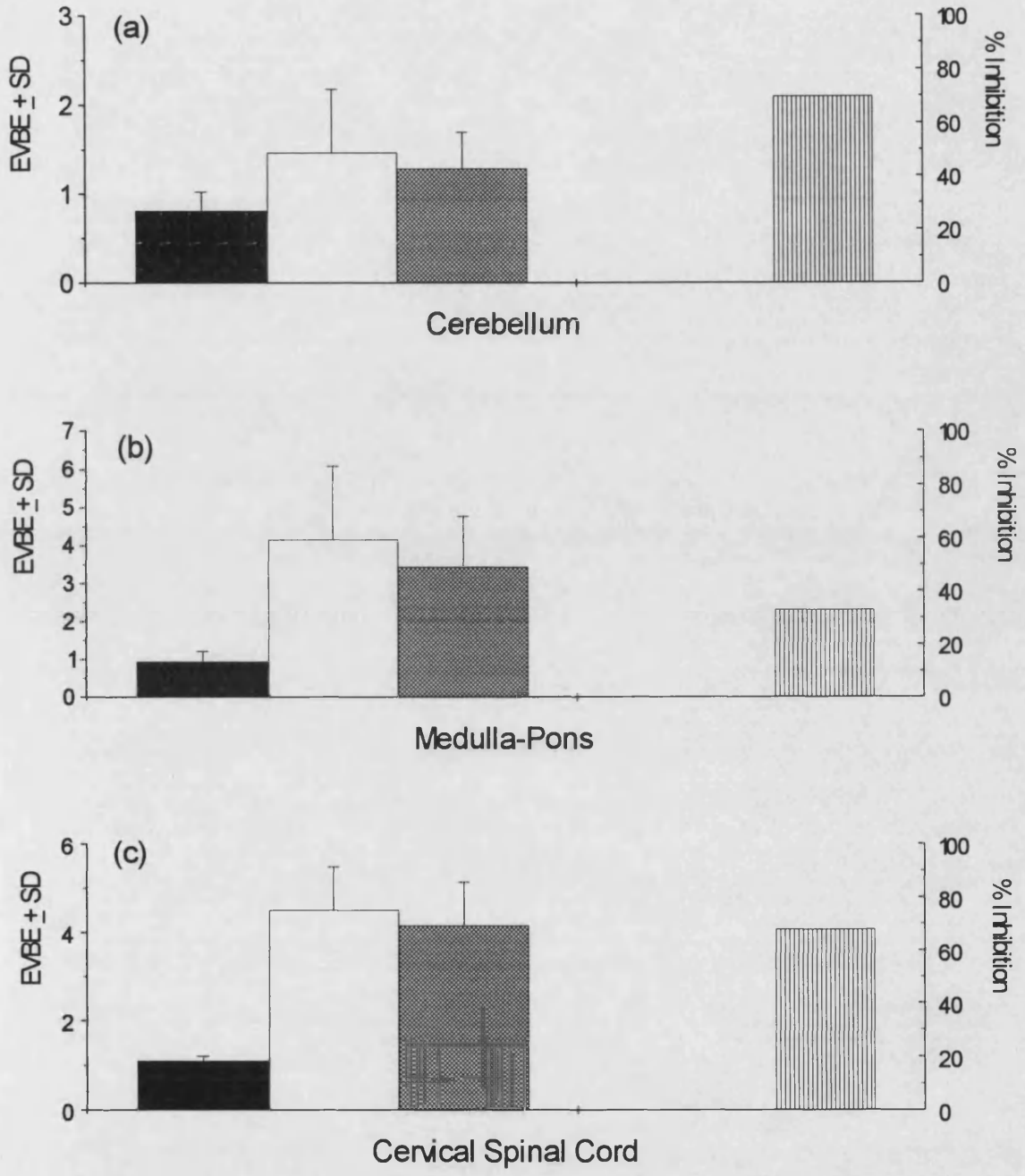


Figure 38: 0.1mg/kg DEX and 25mg/kg CSA, Combined Dose during the Acute Phase of EAE. Histograms show the effect of combined 0.1mg/kg DEX and 25mg/kg CSA administration on BBB restoration in **(a)** cerebellum, **(b)** medulla-pons and **(c)** cervical spinal cord tissues. Results are shown for normal (black column, n = 6), vehicle (open column, n = 5) and combined drug (cross hatch column, n = 8) groups. No significant difference between vehicle and combined drug treated mean EVBE values was shown; Mann-Whitney U. The far right-hand column (vertical hatch) illustrates the calculated additive effect of the two drug concentrations, taken from the differences between drug and vehicle means in the single drug studies. The combined drug effect does not exceed the predicted value.

Table 9: Summary of Neurological Deficits, Plasma Creatinine and Weight Loss following Joint Administration of CSA and DEX

Group	n	MNS ± SD[b]	% Weight Loss ± SD[c]	[Creatinine] mg/dL ± SD[d]
0.01mg/kg DEX[a]	5	2.80 ± 1.79	9.7 ± 2.1	-
0.1mg/kg DEX[a]	6	1.33 ± 1.00*	8.6 ± 2.7	-
25mg/kg CSA[a]	5	1.20 ± 1.60	7.5 ± 3.8	-
35mg/kg CSA[a]	6	0.17 ± 0.40*	8.0 ± 2.8	0.388 ± 0.062
50mg/kg CSA[a]	7	0.20 ± 0.40*	8.7 ± 3.3	-
Normal	3	-	-	0.327 ± 0.063
Vehicle (0.1/25)	6	2.10 ± 1.75	5.6 ± 2.1	-
0.1mg/kg DEX / 25mg/kg CSA	8	0≠	9.5 ± 2.4⊗	-
Vehicle (0.1/35)	5	1.40 ± 0.96	10.7 ± 3.0	0.300 ± 0.038
0.1mg/kg DEX / 35mg/kg CSA	6	0≠	10.8 ± 3.3	0.484 ± 0.095§
Vehicle (0.1/50)	6	2.42 ± 1.50	7.4 ± 2.8	-
0.1mg/kg DEX / 50mg/kg CSA	6	0.08 ± 0.20#	10.5 ± 2.4	-
Vehicle (0.01/50)	8	0.81 ± 1.00	9.9 ± 2.7	-
0.01mg/kg DEX / 50mg/kg CSA	6	0≠	9.4 ± 2.4	-

[a] : Summary of single drug results.* significantly different from respective vehicle controls.

[b] : MNS observed on the day of sampling. # $p < 0.05$ significantly different to vehicle, Mann-Whitney U. ≠ Complete suppression of neurological deficits.

[c] : % difference between body weight prior to disease onset and the day of sampling. ⊗ $p < 0.01$ significantly different to vehicle control, T-test.

[d] : Creatinine analysis was conducted on the combined drug group demonstrating the greatest improvement in pharmacological response at the BBB. § $p < 0.05$ significantly different to vehicle, ANOVA and Tukey's pairwise comparison.

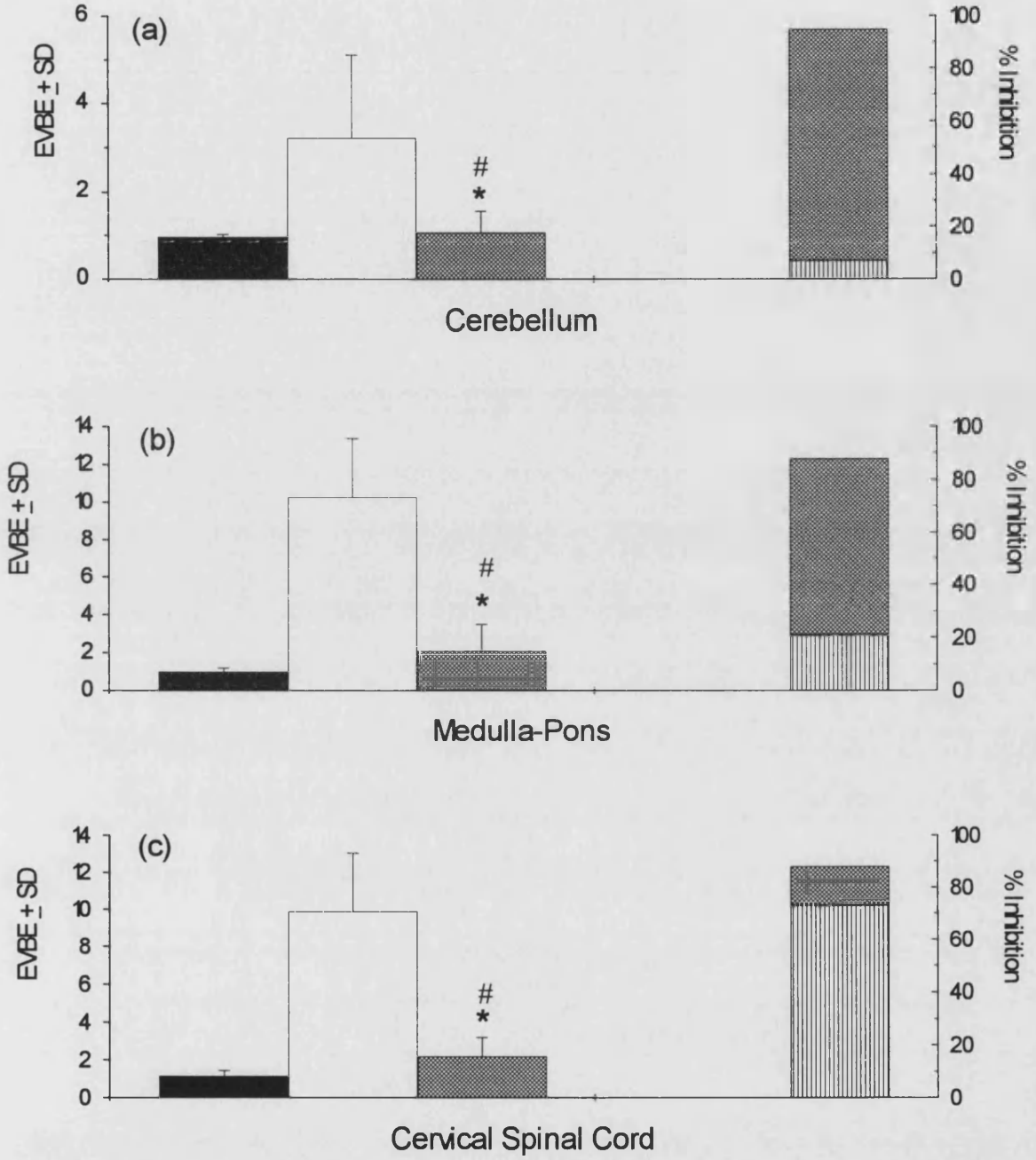


Figure 39: 0.1mg/kg DEX and 35mg/kg CSA, Combined Dose during the Acute Phase of EAE. The histogram shows the effect of combined 0.1mg/kg DEX and 35mg/kg CSA administration on BBB restoration in **(a)** cerebellum, **(b)** medulla-pons and **(c)** cervical spinal cord tissues. Results are shown for normal (black column, n = 4), vehicle (open column, n = 5) and combined drug (cross hatch column, n = 6) groups. Mean EVBE values analysed by Mann-Whitney U found significant differences of * $p < 0.05$ between vehicle and combined drug treatments and # no significant difference between drug and normal groups. The far right-hand column (vertical hatch) illustrates the calculated additive effect of the two drug concentrations taken from the differences between drug and vehicle means in the single drug studies. The combined drug effect (cross hatch) is apparent above the vertically hatched column where the combined drug effect exceeds the predicted value.

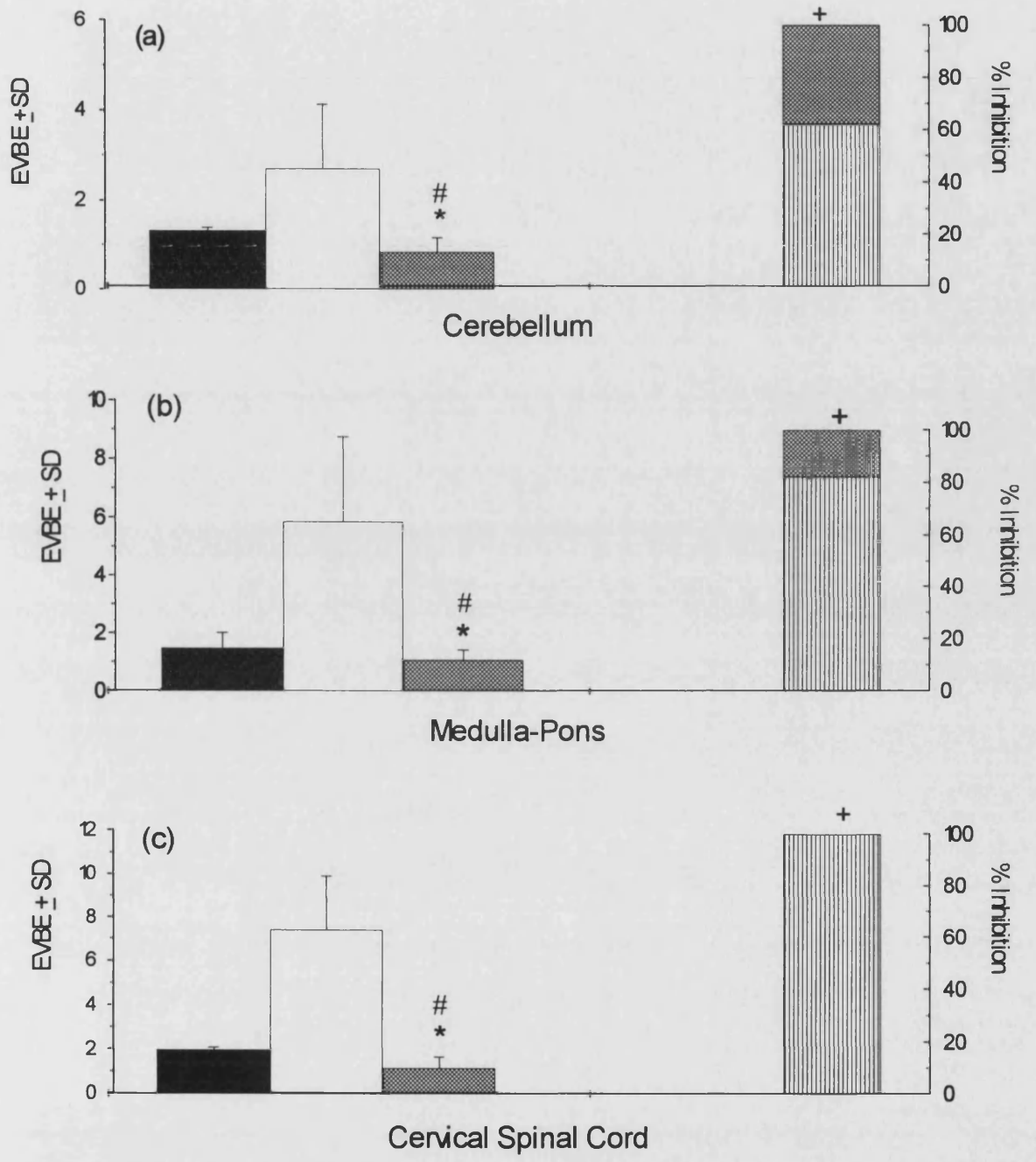


Figure 40: 0.1mg/kg DEX and 50mg/kg CSA, Combined Dose during the Acute Phase of EAE. The histogram shows the effect of combined 0.1mg/kg DEX and 50mg/kg CSA administration on BBB restoration in **(a)** cerebellum, **(b)** medulla-pons and **(c)** cervical spinal cord tissues. Results are shown for normal (black column, n = 3), vehicle (open column, n = 6) and combined drug (cross hatch column, n = 6) groups. Mean EVBE values analysed by Mann-Whitney U found significant differences of * $p < 0.05$ between vehicle and combined drug treatments and # no significant difference between drug and normal groups. The far right-hand column shows the calculated additive effect of the two drug concentrations (vertical hatch) taken from the differences between drug and vehicle means in the single drug studies. The combined drug effect (cross hatch) is apparent above the vertically hatched column where the combined drug effect exceeds the predicted value; + indicates greater than 100% inhibition.

Finally, manipulation of the DEX component to a level ineffective in single drug studies - 0.01mg/kg body weight - was analysed in combination with 50mg/kg body weight CSA. Figure 41 shows a marked reduction in the permeability of the neurovasculature in all tissues ($p < 0.05$), with the barrier function in the C being reduced to within normal limits. Furthermore, the % inhibition measured in the C is greater than the predicted additive effect from the single drug studies (C: single 62%; combined 89%). Augmentation of the single drug effects of CSA can therefore be achieved with doses of DEX as low as 0.01mg/kg body weight.

A study of the log responses against variation in CSA concentration showed a significant linear regression in all areas of the CNS (C, $p = 0.0$, 45% fit; MP, $p = 0.0$, 62% fit; CSp, $p = 0.0$, 71% fit). Similar results were achieved for DEX variation and log response (C, $p = 0.0$, 46% fit; MP, $p = 0.0$, 56% fit; CSp, $p = 0.0$, 69% fit).

Analysis of body weight changes shows a significant difference only between vehicle and 0.1mg/kg DEX and 25mg/kg CSA in combination and is unlikely to be indicative of toxicity as no other drug combination using higher CSA concentrations display the same trend (Table 9). Measurement of plasma creatinine highlighted a significant difference between vehicle and 0.1mg/kg DEX and 35mg/kg CSA in combination, although neither value was shown to be significantly different to normal rat values. Therefore, the improvement in barrier integrity seen with low-dose DEX and CSA in combination is not generally associated with toxic side-effects.

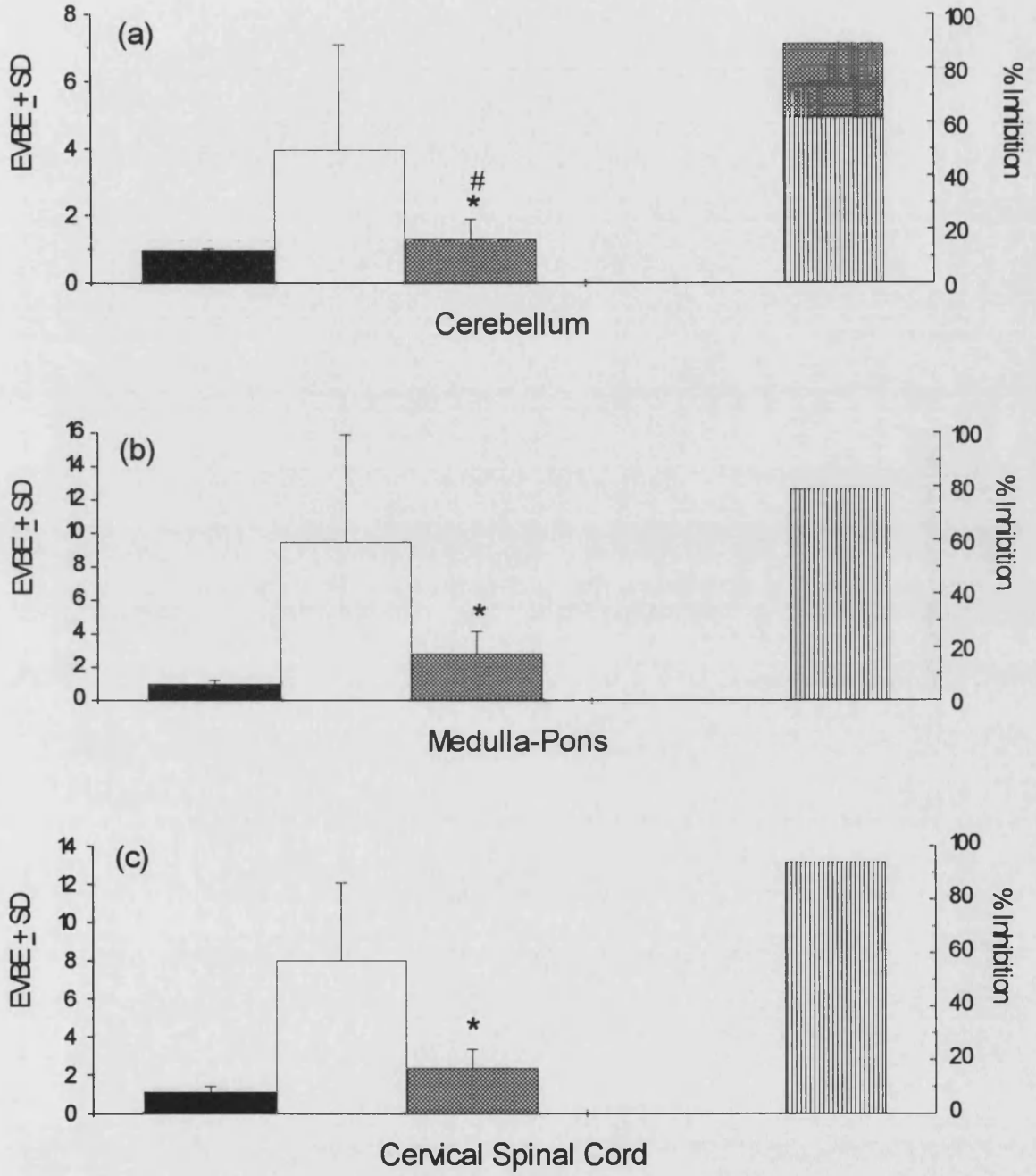


Figure 41: 0.01mg/kg DEX and 50mg/kg CSA Combined Dose during the Acute Phase of EAE. The histogram shows the effect of combined 0.01mg/kg DEX and 50mg/kg CSA administration on BBB restoration in **(a)** cerebellum, **(b)** medulla-pons and **(c)** cervical spinal cord tissues. Results are shown for normal (black column, n = 4), vehicle (open column, n = 8) and combined drug (cross hatch column, n = 6) groups. Mean EVBE values analysed by Mann-Whitney U found significant differences of * $p < 0.05$ between vehicle and combined drug treatments and # no significant difference between drug and normal groups. The far right-hand column shows the calculated additive effect of the two drug concentrations (vertical hatch), taken from the differences between drug and vehicle means in the single drug studies. The combined drug effect (cross hatch) is apparent above the vertically hatched column where the combined drug effect exceeds the predicted value.

7.2 Discussion

The combination of low-dose glucocorticoid and immunosuppressant successfully improved barrier integrity during acute EAE in a dose-dependent manner. Furthermore, at selected doses a marked synergy between the two agents was noted. Indeed, only a limited steroid addition to the CSA dose was required to normalise neurovascular function, a response not seen when CSA was administered alone during the acute phase of disease. By jointly administering DEX and CSA the concentrations could be reduced to a tenth or half - respectively - of the individual effective dose.

The use of CSA as an immunosuppressant can be associated with a number of adverse side-effects. Renal dysfunction, due to a decreased glomeruler filtration rate (Shulman *et. al.*, 1981; Mihatsch *et. al.*, 1988) and hypertension (Rego *et. al.*, 1991; Gerkens *et. al.*, 1989; Brown *et. al.*, 1993) are common during treatment. Indeed, in conditions where CSA is administered to patients with normal CNS function adverse neurological reactions including encephalopathy, cerebral blindness and a diffuse disorder of the white matter determined by MRI, have been noted (Rubin & Kang, 1987; DeGroen *et. al.*, 1987; Davenport *et. al.*, 1988). Withdrawal, or a reduced dose of immunosuppressant is often sufficient to reverse toxic effects. Glucocorticoid administration may also produce detrimental effects particularly when administered in high concentrations for long periods (Stubbs & Morrell, 1973; Lyons *et. al.*, 1988; Villareal *et. al.*, 1996). Importantly, following discontinuation of steroid treatment prolonged suppression of the hypothalamic-pituitary-adrenal axis occurs (Graber *et. al.*, 1965; Calogero *et. al.*, 1990). Furthermore, as immunosuppressants, DEX and CSA both compromise immunological function rendering the patient vulnerable to common and opportunistic infections (Aucott, 1994; Kim & Perfect, 1989; Williams *et. al.*, 1982).

No indication of renal or neurological side-effects was found during the course of CSA or DEX studies in the Lewis model of EAE. Moreover, the work

presented here illustrates that when administered jointly the concentrations of both compounds can be reduced while maintaining corrective control of BBB function.

Interaction between CSA and glucocorticoids may occur at common targets including arachidonic acid metabolism and the ODC cascade (Koenig *et. al.*, 1989b; Ohnishi *et. al.*, 1992; Fidelius *et. al.*, 1984; Marone *et. al.*, 1988). Interestingly, components of the PKC signalling pathway may be regulated by both compounds. Glucocorticoid receptor complexes are thought to interact with the PKC phosphoregulatory cascade in some steroid-activated nuclear transcriptions and the transduction signal 'activator of DNA replication' is CSA-sensitive (Ahima *et. al.*, 1992; Kimball *et. al.*, 1993).

The value of combined steroid and immunosuppressant treatment has been recognised in a number of conditions (Bijlsma *et. al.*, 1986; Alexander *et. al.*, 1992; Leovey *et. al.*, 1993). The present work now indicates that a joint low-dose glucocorticoid and immunosuppressant regime may also be of benefit in controlling BBB dysfunction in neurological disorders such as MS.

FK506 may also be a potential candidate for combined administration with corticosteroids. Indeed, a synergistic effect between low-dose DEX and FK506 has already been demonstrated for steroid-regulated gene expression in plasmids (Ning & Sánchez, 1993). Moreover, DEX suppression of histamine paw edema can be enhanced by pretreatment with FK506 (Oyanagui, 1994). The FK binding proteins 52 and 59 are found in association with heat shock proteins in untransformed mammalian steroid receptor complexes (Peattie *et. al.*, 1992; Tai *et. al.*, 1992). Therefore, FK506 may augment the corrective DEX effect on abnormal BBB function, possibly by upregulating glucocorticoid receptor binding to nuclear sites or by increasing the regulatory sites recognised by the steroid-receptor complex.

Furthermore, combinations of immunosuppressants can demonstrate well tolerated additive if not synergetic actions, without glucocorticoid inclusion (Morris

et. al., 1989; Zeevi *et. al.*, 1987; Strepkowski & Kahan, 1993). Evaluation of further drug interactions may be of value in EAE with a view to future treatment of MS.

8.
The Role of the N-methyl-D-aspartate Subtype of Glutamate Receptor in Neurovascular Dysfunction during Acute EAE

8.1 Results

A number of biological molecules released during inflammation demonstrate vasoactive properties on isolated capillary endothelial cells or in models of inflammatory edema formation. Two such examples are the short-lived NO molecule and the PA spermidine, spermine and putrescine produced as endproducts of the ODC cascade. These compounds have been identified as primary mediators of cerebral vessel disruption in non-immune models of BBB damage (Koenig *et. al.*, 1983; Trout *et. al.*, 1986; and Faraci & Brian, 1994).

Preliminary studies published by Bolton *et. al.* (1994), examined the levels of NO and PA in neurovascular isolates from EAE-sensitised Lewis rats. The results sumarised in Figure 42 and Table 10 show that both NO and PA levels are elevated at the time of disease onset. Moreover, the increase in vasoactive mediator production in EAE coincides with aberrant BBB permeability in identical tissues.

Both NO and PA may be generated following activation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor. Furthermore, NMDA receptor activity has recently been reported at neurovascular locations (Koenig *et. al.*, 1992; Giese *et. al.*, 1995). Therefore, it was of interest to determine whether the NMDA receptor was involved in the development of EAE, in particular the breakdown of neurovascular function.

8.1.1 Suppression of EAE by Administering the NMDA Receptor Antagonist, MK801, during the Effector Phase of Disease Induction

MK801, an *in vivo* active NMDA receptor antagonist, was selected to determine whether control of glutamate receptor activity can modulate EAE development and pathology.

Figure 42: Nitrite levels detected in CNS cytosol preparations by Greiss assay
Permission to present this work was kindly given by Gwen Scott. Cytosol preparations from the C, MP and CSp tissues of normal (open bar), CFA-inoculated (horizontal hatch bar) and EAE-sensitised (cross hatch bar) animals, were analysed by the Greiss Reaction (Green *et. al.*, 1982; Scott *et. al.*, submitted for publication). Results were expressed as μmoles nitrite / mg protein. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to normals, Mann-Whitney U test.

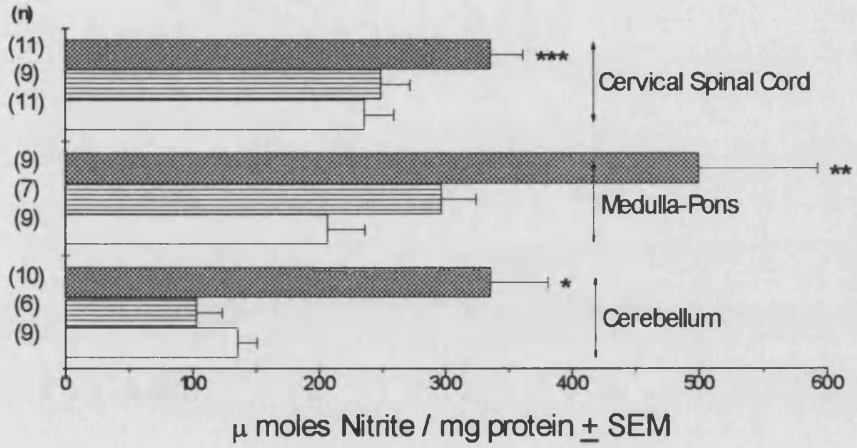


Table 10: Polyamine levels detected in CNS blood vessel isolates
Permission to present this data was kindly given by Pennie Woodyer. Blood vessel isolates were collected from the C, MP and CSp tissues of normal and EAE animals at the height of disease. Polyamine extractions were dansylated and separated by thin-layer chromatography (Seiler, 1983). Assessment of the resultant fluorescent bands was made by densitometry and the findings expressed as a percentage increase above normal values.

Tissue	Polyamine	% Increase compared to normal values
Cerebellum	Spermidine	234
	Spermine	145
	Putrescine	32
Medulla-Pons	Spermidine	177
	Spermine	86
	Putrescine	3
Cervical Spinal Cord	Spermidine	335
	Spermine	224
	Putrescine	38

The first study examined the response of EAE-sensitised animals to the administration of two concentrations of MK801, 0.15 and 0.3mg/kg body weight, from D7-12 PI. The dosing schedule involves the effector phase of disease which follows the ecephalitogenic priming of T cells during the induction stage. Observation of neurological symptoms on D12 PI showed an improved MNS in both 0.15 and 0.3mg/kg treatment groups compared to vehicle controls ($p < 0.05$ and $p < 0.01$ respectively, Table 11). Furthermore, the percentage change in body weight was clearly reduced in both treatment groups, attaining significance in the 0.15mg/kg group ($p < 0.05$, significantly different to vehicle control).

Table 11: Summary of Neurological Signs, Percentage Change in Body Weight and Plasma Corticosterone Levels in EAE-sensitised Lewis rats following MK801 Administration.

Group	n	MNS ± SD[a]	% Weight Change ± SD[b]	n	[Corticosterone] ng/ml ± SD[c]
Normal	-	-	-	8	47.4 ± 26.9
Prophylactic					
Vehicle	10	2.98 ± 1.08	11.7 ± 2.6	10	159.7 ± 115.6
0.15mg/kg MK801	6	1.17 ± 1.47*	5.2 ± 4.8#	6	70.2 ± 19.3
0.3mg/kg MK801	6	0.42 ± 1.02**	6.8 ± 4.8	6	116.0 ± 42.9
Therapeutic					
Vehicle	5	1.08 ± 1.49	7.1 ± 3.0	11	80.7 ± 34.6
0.3mg/kg MK801	6	0.42 ± 0.80	7.3 ± 3.7	8	71.3 ± 28.7
0.6mg/kg MK801	5	0.10 ± 0.22	8.1 ± 3.7	6	67.0 ± 11.1

[a] : MNS recorded on the day of sampling. Prophylactic doses of MK801 significantly reduced MNS, * $p < 0.05$, ** $p < 0.01$; ANOVA with Dunnett's test.

[b] : % Weight change between final body weight and pre-disease weight. # $p < 0.05$, reduction compared to vehicle by 0.15mg/kg dose given prophylactically; ANOVA with Dunnett's test.

[c] : No significant differences between groups; ANOVA.

Administration of MK801 from D7 PI inhibited BBB dysfunction (Figure 43). Low dose MK801 suppressed, but not significantly, neurovascular disruption in all tissues. However, increasing the dose to 0.3mg/kg body weight significantly inhibited the protein extravasation into C, MP and CSp tissues ($p < 0.05$; 0.01; and 0.05 respectively), bringing EVBE values to within normal limits. Analysis of the drug dose-response showed significant linear regression in all tissues (C, $p = 0.002$, 40% fit; MP, $p = 0.008$, 30% fit; CSp, $p = 0.002$, 39% fit).

The compound was found to be well tolerated in an examination of mean body weight profiles (Figure 44a). Indeed, analysis of percentage weight change demonstrated a significant reduction in weight loss for animals receiving low dose MK801 (Table 11).

8.1.2 Suppression of EAE by Therapeutic Administration of the NMDA Receptor Antagonist, MK801

Following the suppression of neurovascular abnormalities and disease symptoms after prophylactic administration of MK801 during the effector phase of EAE, the efficacy of the receptor antagonist was assessed in a therapeutic dosing regime. Doses of 0.3 and 0.6mg/kg body weight were investigated and both concentrations appeared well tolerated by animals following an assessment of mean body weight profiles (Figure 44b).

The glutamate receptor blocker, MK801, demonstrated a restricted control of neurovascular dysfunction when using a dose of 0.3mg/kg (Figure 45). A significant reduction in abnormal permeability was noted in the C and a decreased BBB opening was seen in the MP and CSp. By increasing the dose to 0.6mg/kg body weight a significant restriction of extravasated protein was also recorded in the CSp ($p < 0.05$). The response in the MP did not improve and regression analysis did not demonstrate a significant relationship between dose and log response in the tissue (C, $p = 0.005$, 42% fit; CSp, $p = 0.008$, 38% fit). The development of neurological signs was

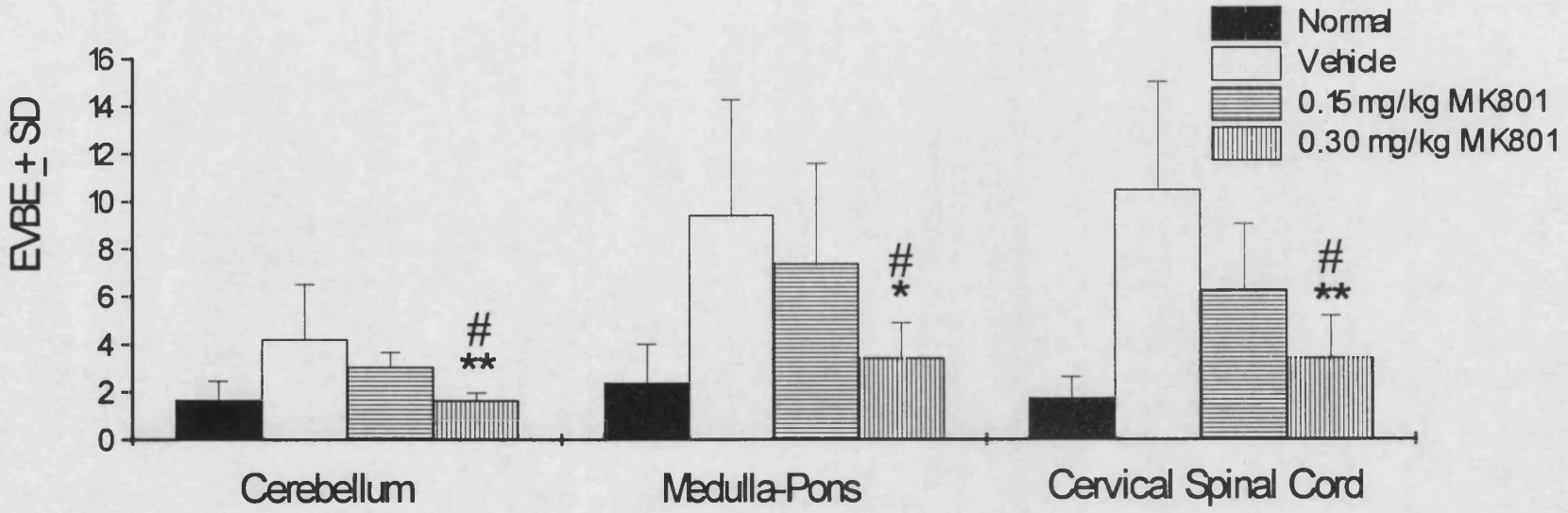


Figure 43: Inhibition of BBB Dysfunction during EAE by Prophylactic Administration of MK801 from D7 PI

MK801 was administered to EAE-sensitised animals for six days beginning on D7 PI and concluding on D12 PI. Examination of BBB permeability to protein was made on D12 PI in normal (black column, n = 6), vehicle (open column, n = 10), 0.15mg/kg MK801 (horizontal hatch column, n = 6) and 0.3mg/kg MK801 (vertical hatch column, n = 6) groups of Lewis rats. * $p < 0.05$, ** $p < 0.01$ compared to vehicle-dosed EAE controls; # no significant difference to normal values; analysis by Mann-Whitney U with Bonferroni correction.

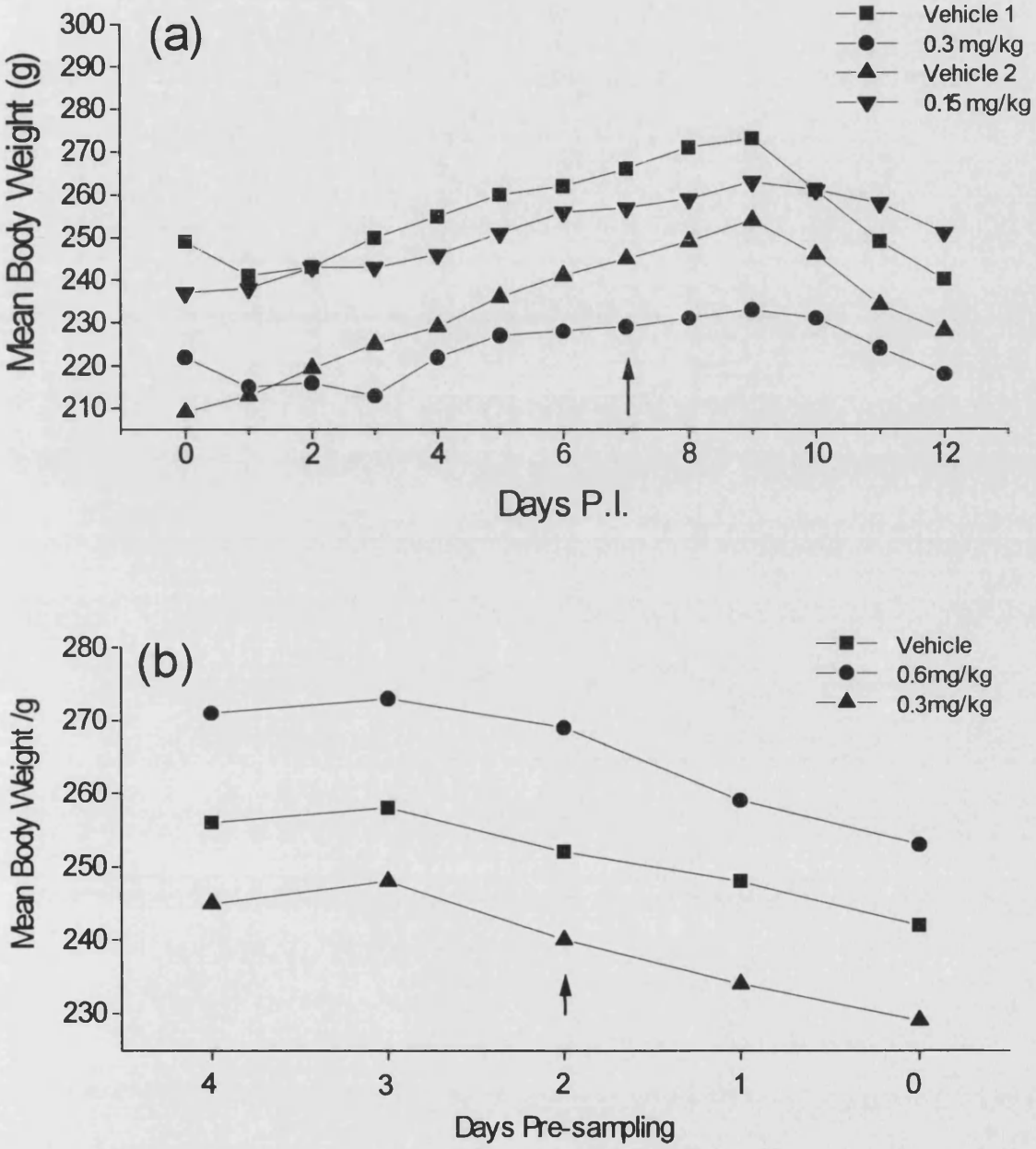


Figure 44: Comparison of Body Weight Profiles from Studies of Prophylactic and Therapeutic MK801 Administration during EAE. Graph **(a)** shows the body weight profiles of 0.15 and 0.3mg/kg MK801 treated groups and vehicle controls (2 and 1 respectively) from each study. Graph **(b)** shows the body weight profiles of 0.3 and 0.6mg/kg MK801 treated groups and vehicle controls from four days pre-sampling. The arrow indicates initiation of the dosing schedule in both graphs.

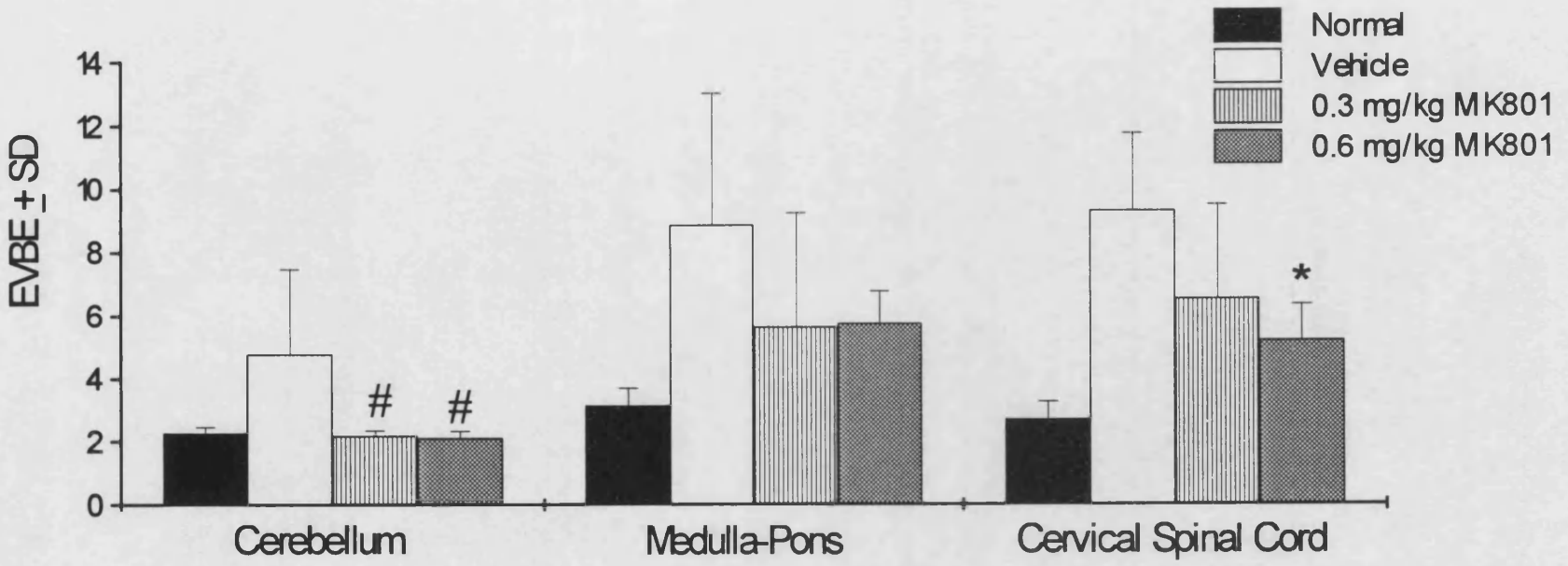


Figure 45: Inhibition of BBB Dysfunction during EAE by Therapeutic Administration of MK801 from Weight Loss
MK801 was administered to EAE-sensitised animals for three days beginning on the day of weight loss and concluding on the day of sampling. Examination of BBB permeability to protein was made in normal (black column, n = 6), vehicle (open column, n = 5), 0.3mg/kg MK801 (vertical hatch column, n = 6) and 0.6mg/kg MK801 (cross hatch column, n = 5) groups of Lewis rats. * $p < 0.05$ compared to vehicle-dosed EAE controls; # no significant difference to normal values; analysis by Mann-Whitney U with Bonferroni correction.

reduced in treatment groups but not found to differ from the MNS range of the vehicle dosed animals (Table 11).

Assessment of endogenous plasma steroid concentration found no significant variation in MK801 dosed groups compared to vehicle controls (Table 11). The receptor antagonist is not therefore generating the effects seen in EAE through a non-specific elevation of corticosterone.

8.2 Discussion

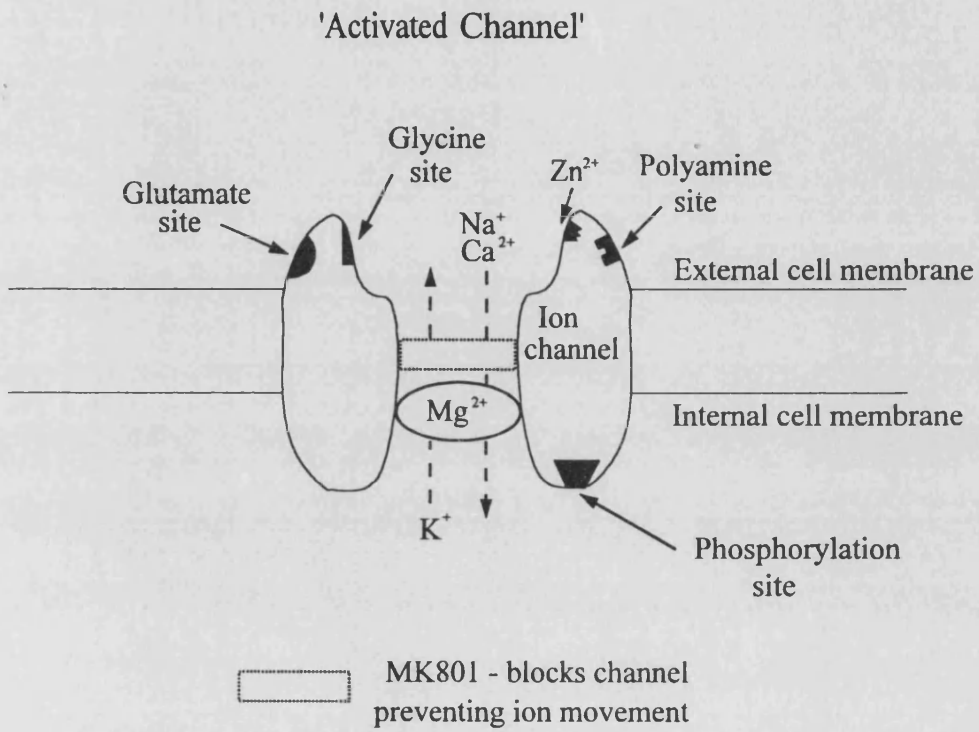
The work shown is the first report describing the suppressive effects of MK801 on pathological features characteristic of EAE. MK801 antagonises the actions of the NMDA-activated glutamate receptor by blocking the ligand-gated Ca^{2+} ion channel (Iversen, 1994). Prophylactic administration of MK801 reduced the development of neurological signs of EAE and significantly suppressed BBB breakdown. Furthermore, short-term therapeutic administration of the antagonist was also able to restrict neurovascular opening. Evaluation of MK801 effects in the development of EAE has reconfirmed the benefits of the double radioisotope technique in assessing the specific actions of novel compounds in the disease.

While this is the first study showing that control of NMDA-receptor activation can modify aspects of EAE, inhibition of glutamate-receptor activity has been demonstrated to be beneficial in other neurological conditions. Neuroprotection by antagonists of this subtype of glutamate receptor have been reported in animal models of focal cerebral ischaemia (Boast *et. al.*, 1988; Park *et. al.*, 1988; Belayev *et. al.*, 1995), closed head trauma (Shapira *et. al.*, 1993) and cold lesion injury (Koenig *et. al.*, 1992). Furthermore, NMDA receptor-mediated neuronal injury by glutamate excitotoxicity is implicated in neurodegenerative conditions including Parkinson's (Zeevalk *et. al.*, 1994) and Alzheimer's disease (Le *et. al.*, 1995).

The effectiveness of MK801 in reducing neurovascular abnormalities and neurological deficits implicates NMDA receptor overactivation in the pathological events underlying EAE. Endogenous upregulation of NMDA receptor activity may be exerted at a number of ligand binding sites (Figure 46), including those of glutamate and glycine (Grimwood *et. al.* ,1993). Interestingly, Honegger *et. al.* (1989b) measured elevated glycine concentrations in the CNS of EAE-diseased animals and found levels 200% greater than control values. Furthermore, Reinhard and co-workers noted that quinolinic acid, an endogenous NMDA receptor agonist and

Figure 46: NMDA receptor

Recognised endogenous ligands and ion binding sites on the NMDA-subtype of glutamate receptor. Attachment of the receptor antagonist MK 801 is indicated .



neurotoxin formed from tryptophan, was markedly elevated in the spinal cord during paralytic EAE (Reinhard *et. al.*, 1992). However, the ligand was not increased in the brain at the height of disease, correlating with the pattern of BBB breakdown and lesion distribution noted for the timepoint (Leibowitz & Kennedy, 1972; Bolton *et. al.*, 1984b; Reinhard *et. al.*, 1992). Therefore, atypical levels of excitatory amino acids capable of stimulating NMDA-receptor activity can be demonstrated in the CNS of neuroantigenically-sensitised animals. Information regarding glutamate receptor agonist levels in MS brains may soon be available with the advent of proton magnetic resonance spectroscopy (Richards, 1991). Interestingly, the technique has identified an increased glutamine-glutamate signal associated with proliferation in glial tumours, which may prove relevant to the occurrence of overt astrocytic hypertrophy within MS lesions (Confort-Gouny *et. al.*, 1993).

The NMDA subtype of glutamate receptor was until recent years thought to be located solely on neurons. However, through the use of specific antagonists NMDA receptor activity has now been demonstrated on brain capillary endothelial cells (Koenig *et. al.*, 1992; Giese *et. al.*, 1995) and more recently on mast cells (Purcell *et. al.*, 1996). Activation of the NMDA receptor generates a Ca^{2+} influx into the cell, stimulating a series of Ca^{2+}-dependent events. Of prime importance in neurological conditions involving loss of BBB integrity is the generation of NO and PA. Elevated ODC activity and individual PA concentrations have been demonstrated in a variety of experimental brain injury models concomitant with neurovascular damage (Dienel & Cruz, 1984; Fike *et. al.*, 1994; Koenig *et. al.*, 1989a,b). Down-regulation of ODC enzyme activity and leakage of BBB tracers was achieved by administration of MK801 following cold injury in rats, confirming a link between glutamate receptor stimulation and PA elevation (Koenig *et. al.*, 1992). Interestingly, recent studies in acute EAE have demonstrated significant increases in PA putrescine, spermidine and spermine at the height of disease by thin-layer and high-performance

liquid chromatography (Bolton *et. al.*, 1994, Paul *et. al.*, submitted 1996), suggesting a role for PA as mediators of BBB damage.

Targeting of upregulated ODC activity in the CNS has proved to be a viable site for pharmacological intervention in the CNS following cold injury (Trout *et. al.*, 1986) and tissue damage after dialysis probe implantation (Fage *et. al.*, 1993). Interestingly, Purcell *et. al.* (1996) have demonstrated that histamine release from mast cells may be induced by PA. Furthermore, the induction of vasoactive amine release is Ca^{2+}-dependent and may be attenuated by both NMDA receptor antagonists MK801 and ifenprodil (Purcell *et. al.* ,1996).

NO is a potent vasodilator which performs a variety of normal signalling functions when produced in picogram quantities. Preliminary studies in EAE have demonstrated the significant elevation of NO product nitrite in cerebral blood vessel isolates at the time of disease expression (Bolton *et. al.*, 1994; Scott *et. al.*, 1994). Further evidence of reactive nitrogen and oxygen species production in acute and hyperacute EAE has been reported by Honegger *et. al.* (1989a) and MacMicking *et. al.* (1992). Indeed, administration of the NO synthase inhibitor aminoguanidine has been observed to suppress both the duration and severity of EAE (Zhao *et. al.*, 1996). Non-disease models of BBB opening and cerebral edema also demonstrate concomitant increases of NO (Mayhan, 1995), which may be attenuated by antioxidant administration (Zuccarello *et. al.*, 1989; Ortego *et. al.*, 1972).

NMDA receptor activation stimulates the upregulation of constitutive NO synthase activity, which is both Ca^{2+} and calmodulin dependent. At inflammatory sites the Ca^{2+}- and calmodulin-independent cytokine-induced NO synthase, which is upregulated in activated macrophages and microglia, is generally considered to be the main source of elevated NO production (Nussler & Billiar, 1993). However, in the cerebral endothelium of EAE-diseased rats both constitutive and inducible NO synthase levels are significantly enhanced in lesion areas (Zhao *et. al.*, 1996). Elevation of NO production via NMDA receptors may therefore account for local

intercellular damage such as dysfunction of BBB transport and mitochondrial deficiencies (Draper & Hibbs, 1988; Packer & Murphy, 1994, 1995).

While NO and PA-mediated BBB damage may result from the activation of endothelial glutamate receptors, the importance of NMDA receptor-derived mediators diffusing from neuronal sites of elevated synthesis should not be discounted. Furthermore, NO is not the only free radical produced following Ca^{2+} channel opening. Superoxide and peroxinitrite also have important roles in neurotoxicity and possibly in EAE and MS (Lafon-Cazal *et. al.*, 1993). Interestingly, following osmotic BBB insult there is an increase in free radical scavenging enzymes, including superoxide dismutase (Schuka *et. al.*, 1993), which rapidly improves the abnormal vascular function. Whether an antioxidant enzyme response is elicited at all, or to a sufficient level to instigate recovery of the damaged neurovasculature of EAE-sensitised animals, has yet to be determined.

The putative role of upregulated NMDA receptor activity in BBB pathology allows further discussion of the sites of action of DEX, CSA and FK506 in correction of neurovascular abnormalities.

DEX consistently suppresses the induction of Ca^{2+} independent NO synthase activity, but Ca^{2+}-dependent NO production is not affected (Radomski *et. al.* ,1990; Palmer *et. al.*, 1992; Geller *et. al.*, 1993; Demerlé-Pallardy *et. al.*, 1993). Elevation of ODC and PA synthesis is under glucocorticoid control and steroid-induced reductions have been demonstrated following cold injury of the neurovasculature (Koenig *et. al.*, 1989b) and in cerebral capillary isolates from treated EAE-sensitised rats (Bolton *et. al.*, 1994). Therefore, glucocorticoids appear to be involved in the regulation of at least one NMDA-receptor stimulated pathway.

As previously mentioned the immunosuppressants FK506 and CSA bind to soluble FK-binding proteins and cyclophillins respectively. The drug-receptor complex then binds to the Ca^{2+}-activated phosphatase, calcineurin, resulting in the inhibition of enzyme activity (Fruman *et. al.*, 1992). NO synthase is a calcineurin

substrate and the abundance of phosphorylated catalitically inactive NO synthase is enhanced by administration of FK506 and CSA (Dawson *et. al.*, 1993). Accumulation of inactive enzyme suggests increased protection against glutamate-induced free-radical toxicity. Manev *et. al.* (1993) confirmed the protective effect of macrolides by preventing NMDA receptor initiated glutamate excitotoxicity in neuronal cell culture. Furthermore, the drug action was shown to be non-immunosuppressive and effective downstream from the site of receptor activation.

Mitochondria are important targets for oxidative damage and disruption of normal energy metabolism may contribute to cellular injury. Indeed, mitochondrial levels at the BBB are reduced during EAE (Claudio *et. al.*, 1989; Oldendorf *et. al.*, 1977) and MS (Claudio, 1995). Elevation of reactive nitrogen and oxygen intermediates within mitochondria cause Ca^{2+} efflux and depolarisation which can be inhibited by the addition of CSA (Packer & Murphy, 1994, 1995). Furthermore, it is proposed that impairment of energy metabolism results in a deterioration of cell membrane function leading to a loss of Mg^{2+} block on NMDA receptors, thereby inducing persistent activation (Turski & Turski, 1993). Therefore, once initiated, mitochondrial aberrations and NMDA-receptor overactivation may be self-perpetuating.

Pharmacological determination of receptor involvement in the loss of neurovascular integrity in EAE has proved invaluable in previous mechanistic investigations. In particular, Goldmuntz, Brosnan and co-workers employed a receptor-specific antagonist to assess the involvement of adrenoreceptors in the loss of neurovascular integrity (Brosnan, 1985,1986; Goldmuntz *et. al.*, 1986; Claudio *et. al.*, 1992). Similarly, MK801 has clearly shown that pharmacological antagonism of the NMDA subtype of glutamate receptor improves the pathology and expression of EAE. MK801 cannot be used in the treatment of related human conditions as it is associated with serious side-effects (Olney *et. al.*, 1989; Tricklebank *et.al.*, 1989). Nevertheless, the prophylactic and therapeutic regimes were well-tolerated in the

current investigation in EAE. Further research into the involvement of NMDA receptor activation in BBB permeability, particularly in EAE, using specific agonist and antagonist combinations, may further understanding of the mechanisms underlying loss of neurovascular integrity. Ultimately, the correction of abnormalities in receptor activation and cellular metabolism may present viable targets for pharmacological intervention in human neuropathologies including the demyelinating diseases.

Conclusions

A double radioisotope technique has been developed from published methodology to quantitate BBB permeability to circulating radiolabelled protein during EAE. The method is highly reproducible demonstrating minimal variation when assessing neurovascular integrity in normal animals. Furthermore, significant differences in BBB abnormalities can be detected between normal and EAE-sensitised rats at the height of disease. The reliability and sensitivity of the technique was repeatedly shown to be suited to the assessment of drug actions on abnormal BBB function.

Increased neurovascular permeability to protein was observed concomitant with the onset of neurological deficits in the Lewis rat model of acute EAE. The dysfunction was maintained throughout the acute episode and continued for a period after symptoms had resolved. The extent of extravasation into individual tissues was time-dependent and matched lesion predilection for CNS areas. However, no correlation between elevated neurovascular permeability and the severity of disease expression could be shown. The abnormal BBB function seen during EAE did not result from non-specific adjuvant effects.

DEX was shown to dose-dependently suppress both neurovascular opening and symptoms of disease when administered in a short-term therapeutic regime, supporting the evidence of steroidal control of barrier dysfunction seen in non-immune mediated models. Glucocorticoid-induced restoration of barrier function was also observed during recovery. Studies with the anti-glucocorticoid RU38486 during acute disease, suggests that DEX is acting through type II glucocorticoid receptors, which suppresses mediators of vascular damage from both CNS and systemic target cells.

The immunosuppressants CSA and FK506, were both effective in inhibiting BBB dysfunction during acute and recovery phases of EAE. Furthermore, results

showed that CSA effected a greater reduction of protein extravasation when administered in the recovery phase. FK506 was equally effective at both timepoints, demonstrating a greater potency than CSA, particularly during the onset of EAE when complete suppression of barrier opening was achieved by the drug. Both drugs have the potential to control vasoactive mediator production by immune and non-immune cells. Further studies are required to determine the actions important to the control of neurovascular abnormalities in EAE.

Combined DEX and CSA treatment in the acute phase of EAE demonstrated highly significant inhibition of neurovascular dysfunction using drug concentrations of minimal individual effectiveness. In selected combinations the response was synergistic. Effective therapies employing reduced concentrations of pharmacological agents in combination may be of benefit to many conditions including neurological disorders such as MS.

The discovery that antagonism of the NMDA receptor elicits a beneficial response in EAE is a unique observation of great significance to the control of the disease. MK801 effectively reduced neurovascular disruption under both prophylactic and therapeutic regimes at a time period when both NO and PA, by-products of NMDA receptor activation, are elevated in untreated EAE animals. The suggested involvement of upregulated glutamate receptor activity in neurovascular perturbation supports similar reports in non-immune models of barrier dysfunction. A pivotal role for the NMDA-receptor in the development of BBB breakdown during EAE would reveal a number of candidate targets for therapeutic intervention at both receptor sites and along subsequent pathways eliciting vasoactive mediators. Ultimately, such findings may have important relevance to the understanding and treatment of BBB dysfunction in human conditions including MS.

Future Directions

Implication of a role for the ionotropic NMDA subtype of glutamate receptor activity and downstream calcium-dependent mechanisms in the loss of BBB integrity and the development of EAE may offer important targets for therapeutic intervention in human conditions such as MS. Therefore the function of the NMDA receptor in neurovascular disruption during EAE requires clarification.

Firstly, neurovascular expression of the glutamate receptor needs to be confirmed in the Lewis rat under normal and diseased conditions. Immunohistochemical staining with antibodies specific for the NR1 subunit, which is an integral component of NMDA receptors, will demonstrate by light microscopy whether these receptors are located in neurovascular regions. The complex nature of the cellular arrangement at the BBB would necessitate the use of electron microscopy with immunogold labelling to identify whether receptor expression was at the endothelial layer, on pericytes or located on glial processes. Further studies employing double antibody labelling against the NMDA receptor subunits and an enzyme such as Na^+-K^+-ATPase known to be polarly distributed at the BBB could identify whether the receptor was selectively expressed on either the luminal or abluminal membranes.

A knowledge of receptor location is of primary importance for the successful antagonism of receptor stimulation and subsequent upregulation of membrane perturbator production. Were findings to show no NMDA receptor expression at the neurovascualture or a purely antiluminal distribution on the endothelial membrane drug studies should be directed towards an analysis of compounds capable of traversing the BBB and reaching neuronal and abluminal sites. Similarly, the action of mast cell-derived NMDA receptors may prove important were no receptors to be located neurovascularly in the Lewis rat. Hence drug administration should coincide with the interaction of mast cells, or mast cell-derived factors, and the neurovasculature leading to abnormal BBB function and progression of EAE.

Following positive identification of NMDA receptor expression at neuroendothelial sites a full characterisation of the subunit composition would confirm whether a single subtype was involved in BBB dysfunction indicating possible links with other NMDA-associated conditions. Furthermore, pharmacological studies could be focused on receptor antagonists with actions against specific subunit combinations such as the NR1/NR2b antagonist ifenprodil.

Once the location and composition of NMDA receptors involved in the breakdown of neurovascular function and progression of EAE has been confirmed the factors controlling overstimulation of receptor function and the subsequent calcium-dependent processes implicated in BBB dysfunction can be studied. *In vitro* analysis of excitatory factor combinations on receptor-induced production of permeability mediators including NO and PA may highlight specific therapeutic targets. Furthermore, the effects of receptor upregulation can be studied on normal BBB characteristics displayed by endothelial cells grown in a glial coculture system, including polar distribution of enzymes and transporters, restricted permeability and a conserved ultrastructure. Successful pharmacological targeting of defined sites *in vitro* may subsequently be analysed in models of EAE by routine neurological, histological and BBB permeability determinations with potential application to human conditions.

References

Acheson, E.D. (1977). Epidemiology of multiple sclerosis. *Br. Med. Bull.* **33**, 9-14.

Adams, C.W.M. (1976). The progression of the lesion in multiple sclerosis. *Neurology* **26**,33-34.(Abstract)

Adams, C.W.M. (1977). Pathology of multiple sclerosis: progression of the lesion. *Br. Med. Bull.* **33**, 15-20.

Adatepe, M., Studer, R., Welch, M. & Potchen, E.J. (1969). Closed system for the preparation of organ specific ^{113m}In compounds. *J. Nucl. Med.* **10**, 318

Ahima, R.S., Garcia, M.M. & Harlan, R.E. (1992). Intracellular localization of corticsteroid receptors in brain: potential interactions with signal transduction pathways. *Proc. Soc. Expt. Biol. Med.* **201**, 244-253.

Alexander, A.G., Barnes, N.C. & Kay, A.B. (1992). Trial of cyclosporin in corticosteroid-dependent chronic severe asthma. *Lancet* **339**, 324-328.

Anders, J.J. & Brightman, M.W. (1979). Assemblies of particles in the cell membranes of developing, mature and reactive astrocytes. *J. Neurocytol.* **8**, 777-795.

Arita, C., Hotokebuchi, T., Miyahara, H., Arai, K., Sugioka, Y., Takagishi, K. & Kaibara, N. (1989). Effect of FK 506 (FR 900506) on collagen arthritis in rats: A preliminary report. *Transplant. Proc.* **21**, 1056-1058.

Arita, C., Hotokebuchi, T., Miyahara, H., Arai, K., Sugioka, Y. & Kaibara, N. (1990). Inhibition by FK506 of established lesions of collagen-induced arthritis in rats. *Clin. exp. Immunol.* **82**, 456-461.

Arya, S.K., Wong-Staal, F. & Gallo, R.C. (1984). Dexamethasone-mediated inhibition of human T cell growth factor and γ-interferon messenger RNA. *J. Immunol.* **133**, 273-276.

Aucott, J.N. (1994). Glucocorticoids and infection. *Endocrinol. Metabol. Clin. North Am.* **23**, 655-670.

Averet, N., Rigoulet, M. & Cohadon, F. (1984). Modifications of synaptosomal Na^+-K^+-ATPase activity during vasogenic brain edema in the rabbit. *J. Neurochem.* **42**, 257-277.

Baker, D., O'Neill, J.K., Gschmeissner, S.E., Wilcox, C.E., Butter, C. & Turk J.L. (1990). Induction of chronic relapsing experimental allergic encephalomyelitis in Biozzi mice. *J. Neuroimmunol.* **28**, 261-270.

Baker, D., O'Neill, J.K. & Turk, J.L. (1991). Cytokines in the central nervous system of mice during chronic relapsing experimental allergic encephalomyelitis. *Cell. Immunol.* **134**, 505-510.

Bakker, D.A. & Ludwin, S.K. (1987). Blood-brain barrier permeability during cuprizone-induced demyelination. Implications for the pathogenesis of immune-mediated demyelinating diseases. *J. Neurol. Sci.* **78**, 125-137.

Barkhof, F., Hommes, O.R., Scheltens, P. & Valk, J. (1991). Quantitative MRI changes in gadolinium-DTPA enhancement after high-dose intravenous methylprednisolone in multiple sclerosis. *Neurology* **41**, 1219-1222.

Barlow, C.F. (1956). A study of abnormal blood-brain barrier permeability in experimental allergic encephalomyelitis. *J Neuropathol Exp Neurol* **15**, 196-208.

Barnes, M.P., Bateman, D.E., Cleland, P.G., Dick, D.J., Walls, T.J., Newman, P.K., Saunders, M. & Tilley, P.J.B. (1985). Intravenous methylprednisolone for multiple sclerosis in relapse. *J Neurol Neurosurg Psychiatry* **48**, 157-159.

Barten, D.M. & Ruddle, N.H. (1994). Vascular cell adhesion molecule-1 modulation by tumor necrosis factor in experimental allergic encephalomyelitis. *J. Neuroimmunol.* **51**, 123-133.

Bastianello, S., Pozzilli, C., Bernardi, S., Bozzao, L., Fantozzi, L.M., Buttinelli, C. & Fieschi, C. (1990). Serial study of gadolinium-DTPA MRI enhancement in multiple sclerosis. *Neurology* **40**, 591-595.

Batchelor, J.R., Compston, D.A.S. & McDonald, W.I. (1978). The significance of the association between HLA and multiple sclerosis. *Br. Med. Bull.* **34**, 279-284.

Baumhefner, R.W., Tourtellotte, W.W., Syndulko, K., Shapshak, P., Merrill, J., Wilson, G.H. & Osborne, M. (1987). Multiple sclerosis: Effect of intravenous natural beta-interferon on clinical neurofunction, magnetic resonance imaging plaque burden, intra-blood-brain barrier IgG synthesis, blood and cerebrospinal fluid cellular immunology, and visual evoked potentials. *Ann Neurol* **22**, 171

Beggs, J.L. & Waggener, J.D. (1976). Transendothelial vesicular transport of protein following compression injury to the spinal cord. *Lab. Invest.* **34**, 428-439.

Begley, D.J., Squires, L.K., Zlokovic, B.V., Mitrovic, D.M., Hughes, C.C.W., Revest, P.A. & Greenwood, J. (1990). Permeability of the blood-brain barrier to the immunosuppressive cyclic peptide cyclosporin A. *J. Neurochem.* **55**, 1222-1230.

Belayev, L., Busto, R., Watson, B.D. & Ginsberg, M.D. (1995). Post-ischemic administration of HU-211, a novel non-competitive NMDA antagonist, protects against blood-brain barrier disruption in photochemical cortical infarction in rats: a quantitative study. *Brain Res.* **702**, 266-270.

Ben-Nun, A., Wekerle, H. & Cohen, I.R. (1981). The rapid isolation of clonable antigen-specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis. *Eur. J. Immunol.* **11**, 195-199.

Betz, A.L., Gilboe, D.D. & Drewes, L.R. (1978). The characteristics of glucose transport across the blood-brain barrier and its relation to cerebral glucose metabolism. In *Transport phenomena in the nervous system* (Levi, G., Battistin, L. and Lajtha, A. eds.), Plenum, New York, pp. 133-149.

Betz, A.L., Firth, J.A. & Goldstein, G.W. (1980). Polarity of the blood-brain barrier: Distribution of enzymes between the luminal and antiluminal membranes of brain capillary endothelial cells. *Brain Res.* **192**, 17-28.

Betz, A.L. & Goldstein, G.W. (1978). Polarity of the blood-brain barrier: Neutral amino acid transport into isolated brain capillaries. *Science* **202**, 225-227.

Betz, A.L. & Goldstein, G.W. (1986). Specialized properties and solute transport in brain capillaries. *Ann. Rev. Physiol.* **48**, 241-250.

Bijlsma, J.W., Schenky, Y., Rameslaar,A.G. & Huber-Bruning, O. (1986). Methylprednisolone pulse therapy in conjunction with azathioprine in rheumatoid arthritis. *Clin. Rheumatol.* **5**, 499-504.

Blackwell, G.J., Flower, R.J., Nijkamp, F.P. & Vane, J.R. (1978). Phospholipase A_2 activity of guinea-pig isolated perfused lungs: stimulation, and inhibition by anti-inflammatory steroids. *Br. J. Pharmacol.* **62**, 79-89.

Bø, L., Olsson, T., Nyland, H., Krüger, P.G., Taule, A. & Mørk, S. (1991). Mast cells in brains during experimental allergic encephalomyelitis in Lewis rats. *J. Neurol. Sci.* **105**, 135-142.

Boast, C.A., Gearharat, S.C., Pastor, E., Lekmann, J. & Etienne, P.E. (1988). The N-methyl-D-aspartate antagonists CGS 19755 and CPP reduce ischemic brain damage in gerbils. *Brain Res.* **442**, 345-348.

Bolton, C., Borel, J.F., Cuzner, M.L., Davison, A.N. & Turner, A.M. (1982). Immunosuppression by cyclosporin A of experimental allergic encephalomyelitis. *J. Neurolog. Sci.* **56**, 147-153.

Bolton, C., Gordon, D. & Turk, J.L. (1984a). Prostaglandin and thromboxane levels in central nervous system tissues from rats during the induction and development of experimental allergic encephalomyelitis (EAE). *Immunopharmacol.* **7**, 101-107.

Bolton, C., Gordon, D. & Turk, J.L. (1984b). A longitudinal study of the prostaglandin content of central nervous system tissues from guinea pigs with acute experimental allergic encephalomyelitis (EAE). *Int. J. Immunopharmac.* **6**, 155-161.

Bolton, C., Turner, A.M. & Turk, J.L. (1984c). Prostaglandin levels in cerebrospinal fluid from multiple sclerosis patients in remission and relapse. *J. Neuroimmunol.* **6**, 151-159.

Bolton, C. (1992). The efficacy of cyclosporin A, FK-506 and prednisolone to modify the adoptive transfer of experimental allergic encephalomyelitis (EAE). *Agents Actions* **35**, 79-84.

Bolton, C., Lees, P., Paul, C., Scott, G.S., Williams, K.I. & Woodyer, P. (1994). Aspects of the biochemical pharmacology of neurovascular disruption in experimental allergic encephalomyelitis (EAE). *J. Neuroimmunol.* **52**, 113 (Abstract).

Bolton, C. & Flower, R.J. (1989). The effects of the anti-glucocorticoid RU 38486 on steroid-mediated suppression of experimental allergic encephalomyelitis (EAE) in the Lewis rat. *Life Sci.* **45**, 97-104.

Booss, J., Esiri, M.M., Tourtellotte, W.W. & Mason, D.Y. (1983). Immunohistological analysis of T lymphocyte subsets in the central nervous system in chronic progressive multiple sclerosis. *J. Neurol. Sci.* **62**, 219-232.

Borel, J.F., Feurer, C., Gubler, H.U. & Stähelin, H. (1976). Biological effects of cyclosporin A: A new antilymphocytic agent. *Agents Actions* **6**, 468-475.

Bornstein, M.B., Miller, A., Slagle, S., Weitzman, M. *et.al.* (1987). A pilot trial of Cop 1 in exacerbating-remitting multiple sclerosis. *N Engl J Med* **317**, 408-414.

Bornstein, M.B. & Crain, S.M. (1965). Functional studies of cultured brain tissues as related to demyelinative disorders. *Science* **148**, 1242-1244.

Bradbury, M.W.B. (1984). The structure and function of the blood-brain barrier. *Federation Proc* **43**, 186-190.

Brett, J., Gerlach, H., Nawroth, P., Steinberg, S., Godman, G. & Stern, D. (1989). Tumour necrosis factor/cachectin increases permeability of endothelial cell monolayers by a mechanism involving regulatory G proteins. *J. Exp. Med.* **169**, 1977-1991.

Brightman, M.W. (1977). Morphology of blood-brain interfaces. *Exp. Eye Res.* **25**, 1-25.

Brightman, M.W. & Reese, T.S. (1969). Junctions between intimately apposed cell membranes in the vertebrate brain. *J. Cell Biol.* **40**, 648-677.

Broadwell, R.D. (1989). Trancytosis of macromolecules through the blood-brain barrier: a cell biological perspective and critical appraisal. *Acta Neuropathol* **79**, 117-128.

Broman, T. (1964). Blood-brain barrier damage in multiple sclerosis: supravital test observations. *Acta Neurol Scand* **40**, 21-24.

Brosnan, C.F., Goldmuntz, E.A., Cammer, W., Factor, S.M., Bloom, B.R. & Norton,W.T. (1985). Prazosin, an α_1-adrenergic receptor antagonist, suppresses experimental autoimmune encephalomyelitis in the Lewis rat. *Proc. Natl. Acad. Sci.* **82**, 5915-5919.

Brosnan, C.F., Sacks, H.J., Goldschmidt, R.G., Goldmuntz,E.A. & Norton W.T. (1986). Prazosin treatment during the effector stage of disease suppresses experimental autoimmune encephalomyelitis in the Lewis rat. *J. Immunol.* **137**, 3451-3456.

Brostoff, S.W. (1977). Immunological responses to myelin and myelin components. In *Myelin* (Morell, P. ed.), Plenum Press, New York, pp. 415-446.

Brostoff, S.W. & Mason, D.W. (1984). Experimental allergic encephalomyelitis: successful treatment in vivo with a monoclonal antibody that recognises T helper cells. *J. Immunol.* **133**, 1938-1942.

Brown, A.L., Wilkinson, R., Thomas, T.H., Levell, N., Munro, C., Marks, J. & Goodship, T.H.J. (1993). The effect of short-term low-dose cyclosporin on renal function and blood pressure in patients with psoriasis. *Br. J. Dermatol.* **128**, 550-555.

Brown, W.J. (1978). The capillaries in acute and subacute multiple sclerosis plaques - a morphometric analysis. *Neurology* **28**, 84-92.

Brown, Z., Neild, G.H. & Lewis, G.P. (1990). Inhibition of prostacyclin formation by cyclosporin is not due to reduced availability of arachidonic acid in membrane phospholipids of cultured human endothelial cells. *Biochem Pharmacol* **39**, 1136-1138.

Burnham, J.A., Wright, R.R., Driesbach, J. & Murray, R.S. (1991). The effect of high-dose steroids on MRI gadolinium enhancement in acute demyelinating lesions. *Neurology* **41**, 1349-1354.

Butter, C., Baker, D., O'Neill, J.K. & Turk, J.L. (1991). Mononuclear cell trafficking and plasma protein extravasation into the CNS during chronic relapsing experimental allergic encephalomyelitis in Biozzi AB/H mice. *J. Neurolog. Sci.* **104**, 9-12.

Calogero, A.E., Kamilaris, T.C., Johnson, E.O., Tartaglia, M.E. & Chrousos, G. (1990). Recovery of the rat hypothalamic-pituitary-adrenal axis after discontinuation of prolonged treatment with the synthetic glucocorticoid agonist dexamethasone. *Endocrinology* **127**, 1574-1579.

Cannella, B. & Raine, C.S. (1995). The adhesion molecule and cytokine profile of multiple sclerosis lesions. *Ann. Neurol.* **37**, 424-435.

Carlos, T.M., Schwartz, B.R., Kovach, N.L., Yee, E., Rosso, M., Osborn, L., Chi-Rosso, G., Newman, B., Lobb, R. & Harlan, J.M. (1990). Vascular cell adhesion molecule-1 mediates lymphocyte adherance to cytokine-activated cultured human endothelial cells. *Blood* **76**, 965-970.

Cerf, J. & Carels, G. (1966). Multiple sclerosis: serum factors producing reversible alterations in bioelectric responses. *Science* **152**, 1066-1068.

Cirillo, R., Triggiani, M., Siri, L., Ciccarelli, A., Pettit, G.R., Condorelli, M. & Marone, G. (1990). Cyclosporin A rapidly inhibits mediator release from human basophils presumably by interacting with cyclophilin. *J. Immunol.* **144**, 3891-3897.

Claudio, L., Kress, Y., Norton, W.T. & Brosnan, C.F. (1989). Increased vesicular transport and decreased mitochondrial content in blood-brain barrier endothelial cells during experimental autoimmune encephalomyelitis. *Am J Pathol* **135**, 1157-1168.

Claudio, L. & Brosnan, C.F. (1992). Effects of prazosin on the blood-brain barrier during experimental autoimmune encephalomyelitis. *Brain Res.* **594**, 233-243.

Claudio, L., Raine, C.S. & Brosnan, C.F. (1995). Evidence of persistent blood-brain barrier abnormalities in chronic-progressive multiple sclerosis. *Acta. Neuropathol.* **90**, 228-238.

Clawson, C.C., Hartmann, J.F. & Vernier, R.L. (1966). Electron microscopy of the effect of gram-negative endotoxin on the blood-brain barrier. *J. Comp. Neurol.* **127**, 183-198.

Clinical biochemistry of domestic animals.(1989) Fourth Edition (Kaneko J.J., Ed) Academic Press

Compston, D.A.S. (1988). Methylprednisolone and multiple sclerosis. *Arch Neurol* **45**, 669-670.

Conde, M., Andrade, J., Bedoya, F.J., Santa Maria, C. & Sobrino, F. (1995). Inhibitory effect of cyclosporin A and FK506 on nitric oxide production by cultured macrophages. Evidence of a direct effect on nitric oxide synthase activity. *Immunology.* **84**, 476-481.

Confort-Gouny, S., Vion-Dury, J., Nicoli, F., Dano, P., Donnet, A., Grazziani N., Gastaut, J.L., Grisoli,F., & Cozzone, P.J. (1993). A multiparametric data analysis showing the potential of localized proton MR spectroscopy of the brain in the metabolic characterization of neurological diseases. *J. Neurolog. Sci.* **118**, 123-133.

Cordon-Cardo, C., O'Brien, J.P., Casals, D., Rittman-Grauer, L., Biedler, J.L., Melamed, M.R. & Bertino, J.R. (1989). Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc. Natl. Acad. Sci. USA* **86**, 695-698.

Cousin, M.A., Lando, D. & Moguilewsky, M. (1982). Ornithine decarboxylase induction by glucocorticoids in brain and liver of adrenalectomized rats. *J. Neurochem.* **38**, 1296-1304.

Crone, C. (1965). Facilitated transfer of glucose from blood into brain tissue. *J. Physiol.* **181**, 103-113.

Crone, C. (1971). The blood-brain barrier - facts and questions. In *Ion homeostasis of the brain* (Siesjö, B.K. and Sorenson, S.C. eds.), Munksgaard, Copenhagen, pp. 52-62.

Crone, C. & Olesen, S.P. (1982). Electrical resistance of brain microvascular endothelium. *Brain Res.* **241**, 49-55.

Cronstein, B.N., Kimmel, S.C., Levin, R.I. & Martiniuk, F. (1992). A mechanism for the antiinflammatory effects of corticosteroids: The glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukoctyte adhesion molecule 1 and intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. USA.* **89**, 9991-9995.

Cserr, H.F. & Knopf, P.M. (1992). Cervical lymphatics, the blood-brain barrier and the immunoreactivity of the brain: a new view. *Immunol. Today* **13**, 507-512.

Culpepper, J.A. & Lee, F. (1985). Regulation of IL-3 expression by glucocorticoids in cloned murine T lymphocytes. *J. Immunol.* **135**, 3191-3197

Cutler, R.W.P., Deuel, R.K. & Barlow, C.F. (1967). Albumin exchange between plasma and cerebrospinal fluid. *Arch Neurol* **17**, 261-270.

Cutler, R.W.P., Lorenzo, A.V. & Barlow, C.F. (1967). Brain vascular permeability to I^{125} gamma globulin and leukocytes in allergic encephalomyelitis. *J Neuropathol Exp Neurol* **26**, 558-571.

Cuzner, M.L., Hayes, G.M., Newcombe, J. & Woodroofe, M.N. (1988). The nature of inflammatory components during demyelination in multiple sclerosis. *J. Neuroimmunol.* **20**, 203-209.

Daëron, M., Sterk, A.R., Hirata, F. & Ishizaka, T. (1982). Biochemical analysis of glucocorticoid-induced inhibition of IgE-mediated histamine release from mouse mast cells. *J. Immunol.* **129**, 1212-1218.

Daniel, P.M., Lam, D.K.C. & Pratt, O.E. (1981). Changes in the effectiveness of the blood-brain and blood-spinal cord barriers in experimental allergic encephalomyelitis. *J. Neurolog. Sci.* **52**, 211-219.

Davenport, A., Will, E.J., Davison, A.M. & Ironside, J.W. (1988). Toxicity of cyclosporin metabolites. *Lancet* **2**, 333

Dawson, T.M., Steiner, J.P., Dawson, V.L., Dinerman, J.L., Uhl, G.R. & Snyder, S.H. (1993). Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate neurotoxicity. *Proc. Natl. Acad. Sci. USA* **90**, 9808-9812.

De Groen, P.C., Aksamit, A.J., Rakela, J., Forbes, G.S. & Krom, R.A.F. (1987). Central nervous system toxicity after liver transplantation: The role of cyclosporine and cholesterol. *N Engl J Med* **317**, 861-866.

De Kloet, E.R., Cousin, M.A., Veldhuis, H.D., Voorhuis, T.D. & Lando, D. (1983). Glucocorticoids modulate the response of ornithine decarboxylase to unilateral removal of the dorsal hippocampus. *Brain Res.* **275**, 91-98.

DeBault, L.E. & Cancilla, P.A. (1980). γ-glutamyl transpeptidase in isolated brain endothelial cells: Induction by glial cells *in vitro*. *Science* **207**, 653-655.

Deguchi, K., Takeuchi, H., Miki, H., Yamada, A., Touge, T., Terada, S. & Nishioka, M. (1991). Effects of FK 506 on acute experimental allergic encephalomyelitis. *Transplant. Proc.* **23**, 3360-3362.

Demerlé-Pallardy, C., Lonchampt, M-O., Chabrier, P-E. & Braquet, P. (1993). Nitric oxide synthase induction in glial cells : effect on neuronal survival. *Life Sci.* **52**, 1883-1890.

Desai, S. & Barton, R. (1989). Pharmacological comparison of active and passive experimental allergic encephalomyelitis in the rat. *Agents Actions* **27**, 351-355.

Dienel, G.A. & Cruz, N.F. (1984). Induction of brain ornithine decarboxylase during recovery from metabolic, mechanical, thermal, or chemical injury. *J. Neurochem.* **42**, 1053-1061.

Dijkstra, C.D., Polman, C.H. & Berkenbosch, F. (1993). Multiple sclerosis: some possible therapeutic opportunities. *TiPS* **14**, 124-129.

Dowling, P.C., Bosch, V.V. & Cook, S.D. (1980). Possible beneficial effect of high-dose intravenous steroid therapy in acute demyelinating disease and transverse myelitis. *Neurology* **30**, 33-36.

Drapier, J-C. & Hibbs, J.B. (1988). Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine-dependent inhibition of mitochondrial iron sulphur enzymes in the macrophage effector cells. *J. Immunol.* **140**, 2829-2838.

Durieu-Trautmann, O., Fédérici, C., Créminon, C., Foignant-Chaverot, N., Roux, F., Claire, M., Strosberg, A.D. & Couraud, P.O. (1993). Nitric oxide and endothelin secretion by brain microvessel endothelial cells: regulation by cyclic nucleotides. *J. C. Physiol.* **155**, 104-111.

Dustin, M.L., Rothlein, R., Bhan, A.K., Dinarello, C.A. & Springer, T.A. (1986). Induction by IL-1 and interferon-γ: tissue distribution, biochemistry and function of a natural adherance molecule (ICAM-1). *J. Immunol.* **137**, 245-254.

Dux, E. & Joó, F. (1982). Effects of histamine on brain capillaries: fine structural and immunohistochemical studies after intracarotid infusion. *Exp. Brain Res.* **47**, 252-258.

Ebers, G. & Bulman, D. (1986). The geography of multiple sclerosis reflects genetic susceptibility. *Neurology* **36**, 108

Ebers, G.C. (1994). Treatment of multiple sclerosis. *Lancet* **343**, 275-279.

Ehrlich, P. (1887). *Ther. Monatsh.* **1**, 88

Einstein, E.R., Chao, L., Csejtey, J., Kibler, R.F. & Shapira, R. (1972). Species specificity in response to tryptophan modified encephalitogen. *Immunochemistry* **9**, 73-84.

Ellison, G.W., Myers, L.W., Mickey, M.R., Graves, M.C., Tourtellotte, W.W., Syndulko, K., Holevoet-Howson, M.I., Lerner, C.D., Frane, M.V. & Pettler-Jennings, P. (1989). A placebo-controlled, randomised, double-masked, variable dosage, clinical trial of azathioprine with and without methylprednisolone in multiple sclerosis. *Neurology* **39**, 1018-1026.

Engell, T. (1989). A clinical patho-anatomical study of clinically silent multiple sclerosis. *Acta Neurol Scand* **79**, 428-430.

Fabry, Z., Waldschmidt, M.M., Moore, S.A. & Hart, M.N. (1990). Antigen presentation by brain microvessel smooth muscle and endothelium. *J. Neuroimmunol.* **28**, 63-71.

Fabry, Z., Waldschmidt, M.M., Hendrickson, D., Keinir, J., Love-Homan, L., Takei, F. & Hart, M.N. (1992). Adhesion molecules on murine brain microvascular endothelial cells: expression and regulation of ICAM-1 and Lgp55. *J. Neuroimmunol.* **36**, 1-11.

Fage, D., Carboni, S., Voltz, C., Scatton, B. & Carter, C. (1993). Ornithine decarboxylase inhibition or NMDA receptor antagonism reduce cortical polyamine efflux associated with dialysis probe implantation. *Neurosci. Lett.* **149**, 173-176.

Fahey, J.V., Guyre, P.M. & Munke, A. (1981). Mechanisms of antinflammatory actions of glucocorticoids. *Adv. Inflammation Res.* **2**, 21-51.

Faraci, F.M. & Brian, J. E. (1994) Nitric Oxide and the cerebral-circulation. *Stroke* **25**, 692-703.

Feldman, B.F. (1989). Cerebrospinal fluid. In *Clinical biochemistry of domestic animals* (Kaneko, J. ed.), Academic Press Inc, San Diego, pp. 835-865.

Fidelus, R.K., Laughter, A.H., Twomey, J.J., Taffet, S.M. & Haddox, M.K. (1984). The effect of cyclosporine on ornithine decarboxylase induction with mitogens, antigens and lymphokines. *Transplantation* **37**, 383-386.

Fike, J.R., Gobbel, G.T., Marton, L.J. & Seilhan, T.M. (1994). Radiation brain injury is reduced by the polyamine inhibitor α-difluoromethylornithine. *Radiat. Res.* **138**, 99-106.

Fishman, J.B., Rubin, J.B., Handrahan, J.V., Connor, J.R. & Fine, R.E. (1987). Receptor-mediated trancytosis of transferrin across the blood-brain barrier. *J. Neurosci. Res.* **18**, 299-304.

Flower, R.J., Parente, L., Persico, P. & Salmon, J.A. (1986). A comparison of the acute inflammatory response in adrenalectomised and sham-operated rats. *Br. J. Pharmacol.* **87**, 57-62.

Fojo, A.T., Ueda, K., Slamon, D.J., Poplack, D.G., Gottesman, M.M. & Pastan, I. (1987). Expression of a multidrug-resistance gene in human tumors and tissues. *Proc. Natl. Acad. Sci. USA* **84**, 265-269.

Fretland, D.J., Widomski, D.L., Shone, R.L., Levin, S. & Gaginella, T.S. (1991). Effect of the leukotriene B_4 receptor antagonist, SC-41930, on experimental allergic encephalomyelitis (EAE) in the guinea pig. *Agents Actions* **34**, 172-174.

Freund, J., Stern, E.R. & Pisani, T.M. (1947). Isoallergic encephalomyelitis and radiculitis in guinea pigs after one injection of brain and mycobacteria in water-in -oil emulsion. *J. Immunol.* **57**, 179-194.

Friden, P.M., Walus, L.R., Musso, G.F., Taylor, M.A., Malfroy, B. & Starzyk, R.M. (1991). Anti-transferrin receptor antibody and antibody-drug conjugates cross the blood-brain barrier. *Proc. Natl. Acad. Sci. USA* **88**, 4771-4775.

Fruman, D.A., Klee, C.B., Bierer, B.E. & Burakoff, S.J. (1992). Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A. *Proc. Natl. Acad. Sci. USA* **89**, 3686-3690.

Gallego, M.J., Farre, A.L., Riesco, A., Monton, M., Grandes, S.M., Barat, A., Hernando, L., Casado, S. & Caramelo, C.A. (1993). Blockade of endothelium-dependent responses in consious rats by cyclosporin A: effect of L-arginine. *Am. J. Physiol. (Heart Circ. Physiol.)* **264**, H708-H714.

Geller, D.A., Nussler, A.K., Silvio, M.D., Lowenstein, C.J., Shapiro, R.A., Wang, S.C., Simmons, R.L. & Billiar, T.R. (1993). Cytokines, endotoxin and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. *Proc. Natl. Acad. Sci. USA* **90**, 522-526.

Gerkens, J.F. (1989). Cyclosporine treatment of normal rats produces a rise in blood pressure and decreased renal vascular responses to nerve stimulation, vasoconstrictors and endothelium-dependent dilators. *J. Pharmacol. Exp. Ther.* **250**, 1105-1112.

Giese, H., Mertsch, K. & Blasig, I.E. (1995). Effect of MK-801and U83836E on a porcine brain capillary endothelial cell barrier during hypoxia. *Neurosci. Lett.* **191**, 169-172.

Goldfeld, A.E., Flemington, E.K., Boussiotis, V.A., Theodos, C.M., Titus, R.G., Strominger, J.L. & Speck, S.H. (1992). Transcription of the tumor necrosis factor α gene is rapidly induced by anti-immunoglobulin and blocked by cyclosporin A and FK506 in human B cells. *Proc. Natl. Acad. Sci. USA* **89**, 12198-12201.

Goldmann, E.E. (1913). Vitalfarbung am Zentralnervensystem. *Abh. Preuss. Akad. Wiss. Phys. Math. Kl.* **1**, 1-60.

Goldmuntz, E.A., Brosnan, C.F. & Norton, W.T. (1986). Prazosin treatment suppresses increased vascular permeability in both acute and passively transferred experimental autoimmune encephalomyelitis in the Lewis rat. *J. Immunol.* **137**, 3444-3450.

Goldstein, G.W. & Betz, A.L. (1986). The blood- brain barrier. *Sci. Am.* **255**, 70-79.

Goodin, D.S. (1991). The use of immunosuppressive agents in the treatment of multiple sclerosis. *Neurology* **41**, 980-985.

Graber, A.L., Ney, R.L., Nicholson, W.E., Island, D.P. & Liddle, G.W. (1965). Natural history of pituitary-adrenal recovery following long-term suppression with corticosteroids. *J. Clin. Endocrinol. Metab.* **25**, 11

Green, L.S., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. & Tannenbaum, S.R. (1982). Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids. *Anal. Biochem.* **126**, 131-138.

Greig, N.H. (1988). Brain tumors and the blood-tumor barrier. In *Implications of the blood-brain barrier and its manipulation* (Neuwelt, E. ed.), Plenum Medical Book Co, New York, pp. 77-106.

Griffiths, R.J., Li, S.W. & Mather, M.E. (1992). Characterisation of passively transferred antigen arthritis induced by methylated bovine serum albumin in the rat: Effect of FK 506 on arthritis development. *Agents Actions* **36**, 146-151.

Grimwood, S., Wilde, G.J.C. & Foster, A.C. (1993). Interactions between the glutamate and glycine recognition sites of the N-methyl-D-aspartate receptor from rat brain, as revealed from radioligand binding studies. *J. Neurochem.* **60**, 1729-1738.

Grossman, R.I., Lisak, R.P., Macchi, P.J. & Joseph, P.M. (1987). MR of acute experimental allergic encephalomyelitis. *AJNR* **8**, 1045-1048.

Grossman, R.I., Braffman, B.H., Brorson, J.R., Goldberg, H.I., Silberberg, D.H. & Gonzalez-Scarano, F. (1988). Multiple Sclerosis: serial study of gadolinium-enhanced MR imaging. *Radiology* **169**, 117-122.

Guseo, A. & Jellinger, K. (1975). The significance of perivascular infiltrates in multiple sclerosis. *J. Neurol.* **211**, 51-60.

Hamada, H. & Tsuruo, T. (1988). Characterization of the ATPase activity of the Mr 170,000 to 180,000 membrane glycoprotein (P-glycoprotein) associated with multidrug-resistance in K562/ADM cells. *Cancer Res.* **48**, 4926-4932.

Harik, S.I. (1992). Changes in the glucose transporter of brain capillaries. *Can. J. Phisiol. Pharmacol.* **70**, 113-117.

Hawkins, C.P., Munro, P.M.G., Mackenzie, F., Kesselring, J., Tofts, P.S., du Boulay, E.P.G.H., Landon, D.N. & McDonald, W.I. (1990). Duration and selectivity of blood-brain barrier breakdown in chronic relapsing experimental allergic encephalomyelitis studied by gadolinium-DTPA and protein markers. *Brain* **113**, 365-378.

Hawkins, C.P., Mackenzie, F., Tofts, P., DuBoulay, E.P.G.H. & McDonald, W.I. (1991). Patterns of blood-brain barrier breakdown in inflammatory demyelination. *Brain* **114**, 801-810.

Hawkins, C.P., Munro, P.M.G., Landon, D.N. & McDonald, W.I. (1992). Metabolically dependent blood-brain barrier breakdown in chronic relapsing experimental allergic encephalomyelitis. *Acta Neuropathol* **83**, 630-635.

Hedley-Whyte, E.T. & Hsu, D.W. (1986). Effect of dexamethasone on blood-brain barrier in the normal mouse. *Ann Neurol* **19**, 373-377.

Helfgott, D.C., May, L.T., Sthoeger, Z., Tamm, I. & Sehgal, P.B. (1987). Bacterial lipopolysaccharide (endotoxin) enhances expression and secretion of β_2 interferon by human fibroblasts. *J. Exp. Med.* **166**, 1300-1309.

Henderson, D.J., Naya, I., Bundick, R.V., Smith, G.M. & Schmidt, J.A. (1991). Comparison of the effects of FK506, cyclosporin A and rapamycin on IL-2 production. *Immunology* **73**, 316-321.

Henning, B., Goldblum, S.E. & McClain, C.J. (1987). Interleukin-1 (IL-1) and tumor necrosis factor/cachectin (TNF) increase endothelial permeability in vitro. *J. Leukoc. Biol.* **42**, 551-552.

Herrmann, H., Neuenfeldt, D., Dittmann, J. & Palleske, H. (1972). The influence of dexamethasone on water content, electrolytes, blood-brain barrier and glucose metabolism in cold injury edema. In *Steroids and brain edema* (Reulen, H.J. and Schürmann, K. eds.), Springer Verlag, New York, pp. 77-86.

Hess, A.D. (1993). Mechanisms of action of cyclosporine: considerations for the treatment of autoimmune diseases. *Clin. Immunol. Immunopathol.* **68**, 220-228.

Hinrichs, D.J., Wegmann, K.W. & Peters, B.A. (1983). The influence of cyclosporin A on the development of actively induced and passively transferred experimental allergic encephalomyelitis. *Cell. Immunol.* 77, 202-209.

Hirata, F., Schiffman, E., Venkatasubramanian, K., Salomon, D. & Axelrod, J. (1980). A phospholipase A_2 inhibitory protein in rabbit neutrophils induced by glucocorticoids. *Proc. Natl. Acad. Sci. USA* 77, 2533-2536.

Hofman, F.M., von Hanwehr, R.I., Dinarello, C.A., Mizel, S.B., Hinton, D. & Merrill, J.E. (1986). Immunoregulatory molecules and IL-2 receptors identified in multiple sclerosis brain. *J. Immunol.* **136**, 3239-3245.

Honegger, C.G., Krenger, W. & Langemann, H. (1989a). Measurement of free radical scavengers in the spinal cord of rats with experimental allergic encephalomyelitis. *Neurosci. Lett.* **98**, 327-332.

Honegger, C.G., Krenger, W. & Langemann, H. (1989b). Changes in amino acid contents in the spinal cord and brainstem of rats with experimental autoimmune encephalomyelitis. *J. Neurochem.* **53**, 423-427.

Hoogstraten, M.C. & Minderhoud, J.M. (1990). Long-term effect of ACTH treatment of relapse in multiple sclerosis. *Acta Neurol Scand* **82**, 74-77.

Hosain, P., Hosain, F., Iqbal, Q.M., Carulli, N. & Wagner, H.N. (1969). Measurement of plasma volume using $^{99}Tc^{m}$, and $^{113}In^{m}$ labelled proteins. *Br. J. Radiol.* **42**, 627-630.

Huber, M., Bamborschke, S., Assheuer, J. & Heiß, W.D. (1988). Intravenous natural beta-interferon treatment of chronic exacerbating-remitting multiple sclerosis: clinical response and MRI/CSF findings. *J. Neurol.* **235**, 171-173.

Hughes, C.C.W., Male, D.K. & Lantos, P.L. (1988). Adhesion of lymphocytes to cerebral microvascular cells: effects of interferon-γ, tumour necrosis factor and interleukin-1. *Immunology* **64**, 677-681.

IFNB MS Study Group. (1993). Interferon beta-1β is effective in relapsing-remitting multiple sclerosis. I. Clinical results of a multicentre, randomised, double-blind, placebo-controlled trial. *Neurology* **43**, 655-661.

Ikeno, T., MacDonnel, P.C., Nagaiah, K. & Guroff, G. (1978). The permissive effect of cortical steroids in the induction of brain ornithine decarboxylase by nerve growth factor. *Biochem. Biophys. Res. Comm.* **82**, 957-963.

Inamura, N., Hashimoto, M., Nakahara, K., Nakajima, Y., Nisho, M., Aoki, H., Yamaguchi, I. & Kohsaka, M. (1988). Immunosuppressive effect of FK506 on experimental allergic encephalomyelitis in rats. *Int. J. Immunopharmac.* **10**, 991-995.

Iversen, L.L. (1994). MK-801 (dizocilpine maleate) - NMDA receptor antagonist. *Neurotransmissions* **10**, 1-4.

Jacobs, C.A., Baker, P.E., Roux, E.R., Picha, K.S., Toivola, B., Waugh, S. & Kennedy, M.K. (1991). Experimental autoimmune encephalomyelitis is exacerbated by IL-1α and suppressed by soluble IL-1 receptor. *J. Immunol.* **146**, 2983-2989.

Jacobs, L., Herndon R., Freeman, A., Cuetter, A., Smith, W.A., Salazar, A.M., Reese, P.A., Josefowicz, R., Husain, F., Ekes, R. & O'Malley, J.A. (1986). Multicentre double-blind study of effect of intrathecally administered natural human fibroblast interferon on exacerbations of multiple sclerosis. *Lancet* **2**, 1411-1413.

James, H. & Fischer, J.E. (1981). Encephalopathy and the blood/brain barrier. *Lancet* **2**, 1420-1421.

Jankovic, B.D., Draskoci, M. & Janjic, M. (1965). Passive transfer of allergic encephalomyelitis with antibrain serum injected into the lateral ventricle of the brain. *Nature* **207**, 428-429.

Janzer, R.C. & Raff, M.C. (1987). Astrocytes induce blood-brain barrier properties in endothelial cells. *Nature* **325**, 253-257.

Jones, H.C. & Keep, R.F. (1987). The control of potassium concentration in the cerebrospinal fluid and brain interstitial fluid of developing rats. *J. Physiol.* **383**, 441-453.

Joó, F. (1985). The blood-brain barrier *in vitro:* Ten years of research on microvessels isolated from the brain. *Neurochem. Int.* **7**, 1-25.

Juhler, M., Barry, D.I., Offner, H., Konat, G., Klinken, L. & Paulson, O.B. (1984). Blood-brain and blood-spinal cord barrier permeability during the course of experimental allergic encephalomyelitis in the rat. *Brain Res.* **302**, 347-355.

Jung-Testas, I., Renoir, M., Bugnard, H., Greene, G.L. & Baulieu, E. (1992). Demonstration of steroid hormone receptors and steroid action in primary cultures of rat glial cells. *J. Steroid Biochem. Mol. Biol.* **41**, 621-631.

Kang, Y.S., Terasaki, T., Ohnishi, T. & Tsuji, A. (1990). *In vivo* and *in vitro* evidence for a common carrier mediated transport of choline and basic drugs through the blood-brain barrier. *J. Pharmacobio-Dyn.* **13**, 353-360.

Kappos, L. (1988). Clinical trials of immunosuppression and immunomodulation in multiple sclerosis. *J. Neuroimmunol.* **20**, 261-268.

Kato, S. & Nakamura, H. (1989). Ultrastructural and ultracytochemical studies on the blood-brain barrier in chronic relapsing experimental allergic encephalomyelitis. *Acta Neuropathol* **77**, 455-464.

Kawai, S., Brandon, D.D., Loriaux, D.L. & Chrousos, G.P. (1989). Interaction of RU 486, a glucocorticoid and progestin antagonist with human circulating mononuclear leukocytes. *Am. J. Med. Sci.* **298**, 167-171.

Kerlero de Rosbo, N., Jago, M.V., Carnegie, P.R. & Bernard, C.C.A. (1987). Correlation of the onset of experimental autoimmune encephalomyelitis-like clinical signs with oedema of the spinal cord in tunicamycin-poisoned rats. *J. Neurolog. Sci.* **78**, 281-294.

Kermode, A.G., Tofts, P.S., Thompson, A.J. MacManus, D.G., Rudge, P., Kendall, B.E., Kingsley, D.P.E., Moseley, I.F., du Boulay E.P.G.H. & McDonald W.I. (1990). Heterogeneity of blood-brain barrier changes in multiple sclerosis: an MRI study with gadolinium-DTPA enhancement. *Neurology* **40**, 229-235.

Kesselring, J., Miller, D.H., MacManus, D.G., Johnson, G., Milligan, N.M., Scolding, N., Compston, D.A.S. & McDonald, W.I. (1989). Quantitative magnetic resonance imaging in multiple sclerosis: the effect of high dose intravenous methylprednisolone. *J Neurol Neurosurg Psychiatry* **52**, 14-17.

Kim, J.H. & Perfect, J.R. (1989). Infection and cyclosporine. *Rev. Infect. Dis.* **11**, 677-690.

Kimball, P.M., Kerman, R.K., Van Buren, C.T., Lewis, R.M., Katz, S. & Kahan, B.D. (1993). Cyclosporine and rapamycin affect protein kinase C induction of the intracellular activation signal, activator of DNA replication. *Transplantation* **55**, 1128-1132.

Kitamura, Y., Itano, Y., Kubo, T. & Nomura, Y. (1994). Suppressive effect of FK506, a novel immunosuppressant, against MPTP-induced dopamine depletion in the striatum of young C57BL/6 mice. *J. Neuroimmunol.* **50**, 221-224.

Koenig, H., Goldstone, A.D. & Lu, C.Y. (1983). Blood-brain barrier breakdown in brain edema following cold injury is mediated by microvascular polyamines. *Biochem. Biophys. Res. Comm.* **116**, 1039-1048.

Koenig, H., Goldstone, A.D. & Lu, C.Y. (1989a). Blood-brain barrier breakdown in cold-injured brain is linked to a biphasic stimulation of ornithine decarboxylase activity and polyamine synthesis: Both are coordinately inhibited by verapamil, dexamethasone, and aspirin. *J. Neurochem.* **52**, 101-109.

Koenig, H., Goldstone, A.D. & Lu, C.Y. (1989b). Polyamines mediate the reversible opening of the blood-brain barrier by the intracarotid infusion of hyperosmolal mannitol. *Brain Res.* **483**, 110-116.

Koenig, H., Trout, J.J., Goldstone, A.D. & Lu, C.Y. (1992). Capillary NMDA receptors regulate blood-brain barrier function and breakdown. *Brain Res.* **588**, 297-303.

Koh, C. & Paterson, P.Y. (1987). Suppression of clinical signs of cell-transferred experimental allergic encephalomyelitis and altered cerebrovascular permeability in Lewis rats treated with a plasminogen activator inhibitor. *Cell. Immunol.* **107**, 52-63.

Komarek, A. & Dietrich, F.M. (1971). Chemical prevention of experimental allergic encephalomyelitis in rats: A quantitative evaluation of steroids and various non-steroid drugs. *Arch. int. Pharmacodyn.* **193**, 249-257.

Koopmans, R.A., Li, D.K.B., Oger, J.J.F., Mayo, J. & Paty, D.W. (1989). The lesion of multiple sclerosis: Imaging of acute and chronic stages. *Neurology* **39**, 959-963.

Kreutzberg, G.W. & Tóth, L. (1983). Enzyme cytochemistry of the cerebral microvessel wall. *Acta Neuropathol. (Suppl.)* **8**, 35-41.

Krisch, B., Leonhardt, H. & Buchheim, W. (1978). The functional and structural border of the neurohemal region of the median eminence. *Cell Tissue Res.* **192**, 327-339.

Krisch, B., Leonhardt, H. & Oksche, A. (1984). Compartments and perivascular arrangement of the meninges covering the cerebral cortex of the rat. *Cell Tissue Res.* **238**, 459-474.

Kuchroo, V.K., Sobel, R.A., Laning, J.C., Martin, C.A., Greenfield, E., Dorf, M.E. & Lees, M.B. (1992). Experimental allergic encephalomyelitis mediated by cloned T cells specific for a synthetic peptide of myelin proteolipid protein. *J. Immunol.* **148**, 3776-3782.

Kurtzke, J.F. (1983). Rating neurological impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology* **33**, 1444-1452.

Lafon-Cazal, M., Culcasi, M., Gaven, F., Pietri, S. & Bockaert, J. (1993). Nitric oxide, superoxide and peroxynitrite: putative mediators of NMDA-induced cell death in cerebellar granule cells. *Neuropharmacol.* **32**, 1259-1266.

Lafferty, K.J., Borel, J.F. & Hodgkin, P. (1983). Cyclosporine-A (CsA): models for the mechanism of action. *Transplant Proc.* **15**, 2242-2247.

Lam, D.K.C. (1986). The blood-central nervous system barrier in acute experimental allergic encephalomyelitis. In *The blood-brain barrier in health and disease* (Suckling, A.J., Rumsby, M.G. and Bradbury, M.W.B. eds.), Ellis Horwood, Chichester, England, pp. 158-164.

Lassmann, H., Kitz, K. & Wisniewski, H.M. (1981). *In vivo* effect of sera from animals with chronic relapsing experimental allergic encephalomyelitis on central and peripheral myelin. *Acta Neuropathol* **55**, 297-306.

Lassmann, H., Vass, K., Brunner, C. & Seitelberger, F. (1986). Characterization of inflammatory infiltrates in experimental allergic encephalomyelitis. *Prog. Neuropathol.* **6**, 33-62.

Le, W-D., Colom, L.V., Xie, W., Smith, R.G. Alexianu, M. & Appel, S.H. (1995). Cell death induced by β-amyloid 1-40 in MES 23.5 hybrid clone: the role of nitric oxide and NMDA-gated channel activation leading to apoptosis. *Brain Research.* **686**, 49-60

Lee, S.W., Tsou, A-P., Chan, H., Thomas, J., Petrie, K., Eugui, E.M. & Allison, A.C. (1988). Glucocorticoids selectively inhibit the transcription of the interleukin 1 β gene and decrease the stability of the interleukin 1 β mRNA. *Proc. Natl. Acad. Sci. USA* **85**, 1204-1208.

Leibowitz, S. (1969). Cerebral vascular permeability in experimental allergic encephalomyelitis. *Neuropat. Pol.* **7**, 303-309.

Leibowitz, S. & Kennedy, L. (1972). Cerebral vascular permeability and cellular infiltration in experimental allergic encephalomyelitis. *Immunology* **22**, 859-869.

Leonard, J.P., Mackenzie, F.J., Patel, H.A. & Cuzner, M.L. (1991). Hypothalmic noradrenergic pathways exert an influence on neuroendocrine and clinical status in experimental autoimmune encephalomyelitis. *Brain Behav. Immunity* **5**, 328-338.

Leovey, A., Bako, G., Szabo, J., Kalman, K. & Forizs, E.(1993). Combined Cyclosporin A and methylprednisolone treatment of Graves opthalmopathy. *Acta. Medica. Hungarica* **49**, 179-185.

Levine, S., Simon, J. & Wenk, E.J. (1966). Edema of the spinal cord in experimental allergic encephalomyelitis. *Proc. Natl. Acad. Sci. USA* **123**, 539-541.

Levine, S. & Torrelio, M. (1977). Cerebral inflammation and edema: A model in rats for testing antiinflammatory drugs. *Exp. Neurol.* **56**, 361-369.

Lew, W., Oppenheim, J.J. & Matsushima, K. (1988). Analysis of the suppression of IL-1α and IL-1β production in human peripheral blood mononuclear adherent cells by a glucocorticoid hormone. *J. Immunol.* **140**, 1895-1902.

Likosky, W.H., Fireman, B., Elmore, R., Eno, G., Gale, K., Goode, G.B., Ikeda, K., Laster, J., Mosher, C., Rozance, J., Richmon, J., Rosenberg, S., Samman, A., Sternbach, R., Whaley, J. & Fehrenbacher, L. (1991). Intense immunosuppression in chronic progressive multiple sclerosis: the Kaiser study. *J Neurol Neurosurg Psychiatry* **54**, 1055-1060.

Linington, C., Berger, T., Perry, L. Weerth, S., Hinze-Selch, D., Zhang, Y., Lu, H-C, Lassman,H. & Wekerle, H. (1993). T cell specific for the myelin oligodendrocyte glycoprotein (MOG) mediate an unusual autoimmune inflammatory response in the central nervous system. *Eur. J. Immunol.* **23**, 1364-1372.

Liu, J. (1993). FK506 and cyclosporin, molecular probes for studying intracellular signal transduction. *Immunol. Today* **14**, 290-295.

Long, J.B. & Holaday, J.W. (1985). Blood-brain barrier: Endogenous modulation by adrenal-cortical function. *Science* **227**, 1580-1583.

Long, D.M., Maxwell, R.E. & French, L.A. (1972). The effects of glucosteroids upon experimental brain edema. In *Steroids and brain edema* (Reulen, H.J. and Schürmann, K. eds.), Springer Verlag, New York, pp. 65-76.

Lyons, P.R., Newman, P.K. & Saunders, M. (1988). Methylprednisolone therapy in multiple sclerosis: a profile of adverse effects. *J Neurol Neurosurg Psychiatry* **51**, 285-287.

Mackenzie, F.J., Leonard, J.P. & Cuzner, M.L. (1989). Changes in lymphocyte β-adrenergic receptor density and noradrenaline content of the spleen are early indicators of immune reactivity in acute experimental allergic encephalomyelitis in the Lewis rat. *J. Neuroimmunol.* **23**, 93-100.

MacMicking, J.D., Willenborg, D.O., Weidemann, M.J., Rockett, K.A. & Cowden, W.B. (1992). Elevated secretion of reactive nitrogen and oxygen intermediates by inflammatory leukocytes in hyperacute experimental autoimmune encephalomyelitis: enhancement by the soluble products of encephalitogenic T cells. *J. Exp. Med.* **176**, 303-307.

MacPhee, I.A.M., Antoni, F.A. & Mason, D.W. (1989). Spontaneous recovery of rats from experimental allergic encephalomyelitis is dependent on regulation of the immune system by endogenous adrenal corticosteroids. *J. Exp. Med.* **169**, 431-445.

Manev, H., Faveron, M., Candeo, P., Fadda, E., Lipartiti, M. & Milani, D. (1993). Macrolide antibiotics protect neurons in culture against the N-methyl-D-aspartate (NMDA) receptor-mediated toxicity of glutamate. *Brain Res.* **624**, 331-335.

Marone, G., Triggiani, M., Cirillo, R., Giacummo, A., Siri, L. & Condorelli, M. (1988). Cyclosporin A inhibits the release of histamine and peptide leukotriene C4 from human lung mast cells. *Ricerca Clin. Lab.* **18**, 53-59.

Mason, D., MacPhee, I. & Antoni, F. (1990). The role of the neuroendocrine system in determining genetic susceptibility to experimental allergic encephalomyelitis in the rat. *Immunology* **70**, 1-5.

Mauch, E., Kornhuber, H.H., Pfrommer, U., Hähnel, A., Laufen, H. & Krapf, H. (1989). Effective treatment of chronically progressive multiple sclerosis with low-dose cyclophosphamide with minor side-effects. *Eur. Arch. Psychiatr. Neurol. Sci.* **238**, 115-117.

Mayhan, W.G. (1995). Role of nitric oxide in disruption of the blood-brain barrier during acute hypertension. *Brain Res.* **686**, 99-103.

McAlpines multiple sclerosis (1985). Pathology of multiple sclerosis (Matthews, W.B. ed.), Churchill Livingstone, London, pp. 301-343.

McConahey, P.J. & Dixon, F.J. (1980). Radioiodination of proteins by the use of the Chloamine-T method. In *Methods in Enzymology* vol.**70** (van Vunakis, H. and Langone, J.J. eds.), Academic Press, London, pp. 210-213.

McDonald, W.I. & Barnes, D. (1989). Lessons from magnetic resonance imaging in multiple sclerosis. *TINS* **12**, 376-379.

McDonald, W.I. & Halliday, A.M. (1977). Diagnosis and classification of multiple sclerosis. *Br. Med. Bull.* **33**, 4-8.

McFarlin, D.E. & McFarland, H.F. (1982a). Multiple sclerosis. *N Engl J Med* **307**, 1183-1188.

McFarlin, D.E. & McFarland, H.F. (1982b). Multiple sclerosis. *N Engl J Med* **307**, 1246-1251.

McLean, B.N., Zeman, A.Z.J. & Thompson, E.J. (1993). Patterns of blood-brain barrier impairment and clinical features in multiple sclerosis. *J Neurol Neurosurg Psychiatry* **56**, 356-360.

Medawar, P.B. (1948). Immunity to homologous grafted skin. III. The fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. *Br. J. Exp. Pathol.* **29**, 58-69.

Meyer, J.S. (1985). Biochemical effects of corticosteroids on neural tissues. *Physiological reviews.* **65**, 946-1020.

Mihatsch, M.J., Bach, J.F., Coovadia, H.M., *et.al.* (1988). Cyclosporin-associated nephropathy in patients with autoimmune diseases. *Klin. Wochenschr.* **66**, 43-7.

Milanese, C., La Mantia, L., Salmaggi, A., Campi, A., Eoli, M., Scaioli, V., Nespolo, A. & Corridori, F. (1989). Double-blind randomized trial of ACTH versus dexamethasone versus methylprednisolone in multiple sclerosis bouts. *Eur Neurol* **29**, 10-14.

Milanese, C., Salmaggi, A., La Mantia, L., Campi, A., Eoli, M., Savoiardo, M., Bianchi, G. & Nespolo, A. (1990). Double blind study of intrathecal beta-interferon in

multiple sclerosis: clinical and laboratory results. *J Neurol Neurosurg Psychiatry* **53**, 554-557.

Milhorat, T.H., Davis, D.A. & Lloyd, B.J. (1973). Two morphologically distinct blood-brain barriers preventing entry of cytochrome c into cerebrospinal fluid. *Science* **180**, 76-78.

Miller, D.H., Thompson, A.J., Morrisey, S.P., MacManus, D.G., Moore, S.G., Kendall, B.E., Moseley, I.F. & McDonald, W.I. (1992). High dose steroids in acute relapses of multiple sclerosis: MRI evidence for a possible mechanism of therapeutic effect. *J Neurol Neurosurg Psychiatry* **55**, 450-453.

Milligan, N.M., Newcombe, R. & Compston, D.A.S. (1987). A double-blind controlled trial of high dose methylprednisolone in patients with multiple sclerosis: 1. clinical effects. *J Neurol Neurosurg Psychiatry* **50**, 511-516.

Morris, R.E., Hoyt, E.G., Murphy, M.P. & Shorthouse, R. (1989). Immunopharmacology of FK506. *Transplant. Proc.* **21**, 1042-1044.

Müller, F.A.J., Hänny, P.E., Wichmann, W., Fierz, W. & Dürsteler, M.R. (1989). Cerebrospinal fluid immunoglobulins and multiple sclerosis: Correspondence with magnetic resonance imaging and visually evoked potential changes. *Arch Neurol* **46**, 367-371.

Nalasnik, M.A., Todo, S., Murase, N., Gryzan, S., Lee, P., Makowka, L. & Starzi, T.E. (1987). Toxicology of FK506 in the Lewis rat. *Transplant. Proc.* **19**, 89-92.

Neu I.ger, J., Prossiegel, M., Wildfeuer, A., Mehlber, L. & Ruhenstroth-Bauer, G. (1988). Multiple sclerosis: leukotrienes in the spinal fluid. *Munch. Med. Wschr.* **130**, 80-81.

Newman, E.A. (1985). Membrane physiology of retinal glial (Müller) cells. *J. Neurosci.* **5**, 2225-2239.

Ning, Y-M. & Sánchez, E.R. (1993). Potentiation of glucocorticoid receptor-mediated gene expression by the immunophilin ligands FK506 and rapamycin. *J. Biol. Chem.* **268**, 6073-6076.

Nooter, K., Meershoek, B., Spaans, W., Sonneveld, P., Oostrum, R. & Deurloo, J. (1984). Blood and tissue distribution of cyclosporin A after a single oral dose in the rat. *Experientia* **40**, 559-561.

Nussler, A.K. & Billiar, T.R. (1993). Inflammation, immunoregulation, and inducible nitric oxide synthase. *J. Leukoc. Biol.* **54**, 171-178.

O'Neil, J.K., Butter, C., Baker, D., Gschmeissner, S.E., Kraal, G., Butcher, E.C. & Turk, J.L. (1991). Expression of vascular addressins and ICAM-1 by endothelial cells

in the spinal cord during chronic relapsing experimental allergic encephalomyelitis in the Biozzi AB/H mouse. *Immunology* **72**, 520-525.

Ohkawa, T. (1989). Experimental allergic encephalomyelitis. 1. Oligodendroglial cell population in monkey brain at the acute stage. *Acta Pathologica Japonica* **39**, 224-228.

Ohnishi, T., Posner, J.B. & Shapiro, W.R. (1992). Vasogenic brain edema induced by arachidonic acid: Role of extracellular arachidonic acid in blood-brain barrier dysfunction. *Neurosurgery* **30**, 545-551.

Oldendorf, W.H. (1971). Brain uptake of radiolabelled amino acids, amines, and hexoses after arterial injection. *Am. J. Physiol.* **221**, 1629-1639.

Oldendorf, W.H., Cornford, M.E. & Brown, W.J. (1977). The large apparent work capability of the blood-brain barrier: a study of the mitochondrial content of capillary endothelial cells in brain and other tissues of the rat. *Ann Neurol* **1**, 409-417.

Oldendorf, W.H. & Brown, W.J. (1975). Greater number of capillary endothelial mitochondria in brain than in muscle. *Proc. Soc. Expt. Biol. Med.* **149**, 736-738.

Oldendorf, W.H. & Szabo, J. (1976). Amino acid assignment to one of three blood-brain barrier amino acid carriers. *Am. J. Physiol.* **230**, 94-98.

Oldendorf, W.H. & Towner, H.F. (1974). Blood-brain barrier and DNA changes during the evolution of experimental allergic encephalomyelitis. *J Neuropathol Exp Neurol* **33**, 616-631.

Olney, J.W., Labruyere, J. & Price, M.T. (1989). Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs. *Science* **244**, 1360-1362.

Orr, E.L. & Stanley, N.C. (1989). Brain and spinal cord levels of histamine in Lewis rats with acute experimental autoimmune encephalomyelitis. *J. Neurochem.* **53**, 111-118.

Ortega, B.D., Demopoulos, H.B. & Ransohoff, J. (1972). Effect of antioxidants on experimental cold-induced cerebral edema. In *Steroids and brain edema* (Reulen, H.J. and Schürmann, K. eds.), Springer Verlag, New York, pp. 167-175.

Osman, S. & Danpure, H.J. (1987). A simple in vitro method of radiolabelling human erythrocytes in whole blood with ^{113m}In-tropolonate. *Eur J Haematol* **39**, 125-127.

Oyanagui, Y. (1994). Nitric oxide- and hydrogen peroxide-mediated gene expression by glucocorticoids and FK506 in histamine paw edema of mice. *Life Sciences* **55**,177-185.

Packer, M.A. & Murphy, M.P. (1994). Peroxynitrite causes calcium efflux from mitochondria which is prevented by cyclosporin A. *FEBS Letters* **345**, 237-240.

Packer, M.A. & Murphy, M.P. (1995). Peroxynitrite formed by simultaneous nitric oxide and superoxide generation causes cyclosporin-A-sensitive mitochondrial calcium efflux and depolarisation. *Eur. J. Biochem.* **234**, 231-239.

Palestine, A.G., Nussenblatt, R.B. & Chan, C-C. (1985). Cyclosporine penetration into the anterior chamber and cerebrospinal fluid. *Am. J. Ophthalmol.* **99**, 210-211.

Palmer, R.M.J., Bridge, L., Foxwell, N.A. & Moncada, S. (1992). The role of nitric oxide in endothelial cell damage and its inhibition by glucocorticoids. *Br. J. Pharmacol.* **105**, 11-12.

Papadopoulos, N.M., McFarlin, D.E., Patronas, N.J., McFarland, H.F. & Costello, R. (1987). A comparison between chemical analysis and magnetic resonance imaging with the clinical diagnosis of multiple sclerosis. *Am J Clin Pathol* **88**, 365-368.

Pappius, H.M. (1972). Effects of streoids on cold injury edema. In *Steroids and brain edema* (Reulen, H.J. and Schürmann, K. eds.), Springer Verlag, New York, pp. 57-64.

Pappius, H.M. & Wolfe, L.S. (1983). Functional disturbances in brain following injury. Search for underlying mechanisms. *Neurochem. Res.* **8**, 63-72.

Pardridge, W.M. (1991). Advances in cell biology of blood-brain barrier transport. *Sem. Cell Biol.* **2**, 419-426.

Pardridge, W.M. & Mietus, L.J. (1979). Transport of steroid hormones through the rat blood-brain barrier. Primary role of albumin-bound hormone. *J. Clin. Invest.* **64**, 145-154.

Park, C.K., Nehls, D.G., Graham, D.I., Teasdale, G.M. & McCulloch, J. (1988). Focal cerebral ischaemia in the cat: Treatment with the glutamate antagonist MK801 after induction of ischaemia. *J. Cereb. Blood Flow Metab.* **8**, 757-762.

Paterson, P.Y. (1960). Transfer of allergic encephalomyelitis in rats by means of lymph node cells. *J. Exp. Med.* **111**, 119-135.

Paterson, P.Y. (1976). Experimental autoimmune (allergic) encephalomyelitis: Induction, pathogenesis, and suppression. In *Textbook of immunopathology* (Miescher, P.A. and Müller-Eberhard, H.J. eds.), Grune and Stratton, Inc. pp. 179-213.

Paterson, P.Y. (1976). Experimental autoimmune (allergic) encephalomyelitis: Induction, pathogenesis, and suppression. In *Textbook of immunopathology* (Miescher, P.A. and Müller-Eberhard, H.J. eds.), Grune and Stratton, Inc. pp. 179-213.

Paterson, P.Y. (1978). The demyelinating diseases: Clinical and experimental studies in animals and man. In *Immunological diseases* (Samter, M., Alexander, N., Rose, B., Sherman, W.B., Talmage, D.W. and Vaughn, J.H. eds.), Little Brown, Boston, pp. 1400-1435.

Paty, D.W., Li, D.K.B., Duquette, P., Girard, M., Despault, L., Dubois, R., Knobler, R.L. *et. al.* (1993). Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. II. MRI analysis results of a multicentre, randomised, double-blind, placebo-controlled trial. *Neurology* **43**, 662-667.

Paul, C., Scott, G.S., Barbour, M., Seiler, N. & Bolton, C (1996). N-methyl-D-aspartate (NMDA) receptor-mediated events contribute to nerovascular breakdown during experimental allergic encephalomyelitis (EAE). *Immunology* (Submitted).

Peattie, D.A., Harding, M.W., Fleming, M.A., DeCenzo, M.T., Lippke, J.A., Livingstone, D.J. & Benasutti, M. (1992). Expression and characterization of human FKBP52, an immunophilin that associates with the 90-kDa heat shock protein and is a component of steroid receptor complexes. *Proc. Natl. Acad. Sci. USA* **89**, 10974-10978.

Petito, C.K. & Levy, D.E. (1980). The importance of cerebral arterioles in alterations of the blood-brain barrier. *Lab. Invest.* **43**, 262-268.

Pettinelli, C.B. & McFarlin, D.E. (1981). Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after *in vitro* activation of lymph node cells by myelin basic protein: requirement for Lyt $1^+ 2^-$ T lymphocytes. *J. Immunol.* **127**, 1420-1423.

Pober, J.S., Gimbrone, M.A., Lapierre, L.A., Mendrick, D.L., Fiers, W., Rothlein, R. & Springer, T.A. (1986). Overlapping patterns of activation of human endothelial cells by interleukin-1, tumour necrosis factor and immune interferon. *J. Immunol.* **137**, 1893-1896.

Politi, L.E., Rodriguez de Turco, E.B. & Bazan, N.G. (1985). Dexamethasone effect on free fatty acid and diacylglycerol accumulation during experimentally induced vasogenic brain edema. *Neurochem. Pathol.* **3**, 249-269.

Pollitt, E. and Leibel, R.J. (eds.)(1982). *Iron deficiency: Brain and biochemistry and behaviour* , Raven Press, New York,

Poser, C.M. (1992). Multiple sclerosis: Observations and reflections - a personal memoir. *J. Neurolog. Sci.* **107**, 127-140.

del Pozo, E., Elford, P., Perrelet, R., Graeber, M., Casez, J.P. & Modrowski, D. (1992). Prevention of adjuvant arthritis by cyclosporine in rats. *Semin. Arthritis Rheum.* **21**, 23-29.

Prineas, J.W. & Wright, R.G. (1978). Macrophages, lymphocytes, and plasma cells in the perivascular compartment in chronic multiple sclerosis. *Lab. Invest.* **38**, 409-421.

Prosiegel, M., Neu, I., Mallinger, J., Wildfeuer, A., Mehlber, L., Vogl, S., Hoffmann, G. & Ruhenstroth-Bauer, G. (1989). Suppression of experimental autoimmune encephalomyelitis by dual cyclo-oxygenase and 5-lipoxygenase inhibition. *Acta Neurol Scand* **79**, 223-226.

Purcell, W.M., Doyle, K.M., Westgate, C. & Atterwill, C.K. (1996). Characterisation of a functional polyamine site on rat mast cells: association with a NMDA receptor macrocomplex. *J. Neuroimmunol.* **65**, 49-53.

Quagliarello, V.J., Long, W.J. & Scheld, W.M. (1986). Morphologic alterations of the blood-brain barrier with experimental meningitis in the rat. Temporal sequence and role of encapsulation. *J. Clin. Invest.* **77**, 1084-1095.

Radomski, M.W., Palmer, R.M.J. & Moncada, S. (1990). Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. USA* **87**, 10043-10047.

Raine, C.S., Hummelgard, A., Swanson, E. & Bornstein, M.B. (1973). Multiple sclerosis serum induced demyelination in vitro. *J. Neurolog. Sci.* **20**, 127-148.

Raine, C.S. (1983). Multiple sclerosis and chronic relapsing EAE: comparative ultrastructural neuropathology. In *Multiple sclerosis* (Hallpike, J.F., Adams, C.W.M. and Tourtellotte, W.W. eds.), Chapman and Hall Ltd, London, pp. 413-460.

Raine, C.S. (1984). Biology of disease. Analysis of autoimmune demyelination: Its impact upon multiple sclerosis. *Lab. Invest.* **50**, 608-635.

Raine, C.S. & Scheinberg, L.C. (1988). On the immunopathology of plaque development and repair in multiple sclerosis. *J. Neuroimmunol.* **20**, 189-201.

Raine, C.S. & Stone, S.H. (1977). Animal model for multiple sclerosis: chronic experimental allergic encephalomyelitis in inbred guinea pigs. *NY State J. Med.* **77**, 1693

Reed, J.C., Abidi, A.H., Alpers, J.D., Hoover, R.G., Robb, R.J. & Nowell, P.C. (1986). Effect of cyclosporin A and dexamethasone on interleukin-2 receptor gene expression. *J. Immunol.* **137**, 150-154.

Reese, T.S., Feder, N. & Brightman, M.W. (1971). Electron microscopic study of the blood-brain and blood-cerebrospinal fluid barriers with microperoxidase. *J. Neuropath.* **30**, 137-138.

Reese, T.S. & Karnovsky, M.J. (1967). Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J. Cell Biol.* **34**, 207-217.

Rego, A., Vargas, R., Cathapermal, S., Kuwahara, M., Foegh, M.L. & Ramwell, P.W. (1991). Systemic vascular effects of cyclosporin A treatment in normotensive rats. *J. Pharmacol. Exp. Ther.* **259**, 905-915.

Reiber, H., Suckling, A.J. & Rumsby, M.G. (1984). The effect of Freund's adjuvants on blood-cerebrospinal fluid barrier permeability. *J. Neurol. Sci.* **63**, 55-61.

Reiber, H. (1986). Evaluation of blood-cerebrospinal fluid barrier dysfunctions in neurological diseases. In *The blood-brain barrier in health and disease* (Suckling, A.J., Rumsby, M.G. and Bradbury, M.W.B. eds.), Ellis Horwood, Chichester, England, pp. 130-145.

Reiber, H. & Suckling, A.J. (1986). Cyclosporin-A treatment of experimental allergic encephalomyelitis: changes in immunological regulation and blood-CSF barrier function. *J. Neuroimmunol.* **12**, 121-130.

Reinhard, J.F., Flanagan, E.M., Erickson, J.B., Viveros, O.H. & Chang, S.Y. (1992). The neurotoxin quinolinic acid is selectively elevated in spinal cords of rats with experimental allergic encephalomyelitis (EAE). *Society for Neuroscience Abstracts* **18**, 442(Abstract)

Renkin, E.M. (1977). Multiple pathways of capillary permeability. *Circ Res* **41**, 735-743.

Reulen, H.J., Hadjidimos, A. & Schürmann, K. (1972). The effect of dexamethasone on water and electrolyte content on rCBF in perifocal brain edema in man. In *Steroids and brain edema* (Reulen, H.J. and Schürmann, K. eds.), Springer Verlag, New York, pp.239-252.

Richards, T.L. (1991). Proton MR spectroscopy in multiple sclerosis: value in establishing diagnosis, monitoring progression, and evaluating therapy. *Am. J. Roental.* **157**, 1073-1078.

Rivers, T.M. & Schwentker, F.F. (1935). Encephalomyelitis accompanied by myelin destruction experimentally produced in monkeys. *J. Exp. Med.* **61**, 689-702.

Rodriguez, M. (1989). Multiple sclerosis: basic concepts and hypothesis. *Mayo Clin Proc* **64**, 570-576.

Rose, A.S., Kuzma, J.W., Kurtzke, J.F., Namerow, N.S., Sibley, W.A. & Tourtellotte, W.W. (1970). Cooperative study in the evaluation of therapy in multiple sclerosis: ACTH vs. placebo. *Neurology* **20**, 1-59.

Royall, J.A., Berkow, R.L., Beckman, J.S., Cunningham, M.K., Matalon, S. & Freeman, B.A. (1989). Tumour necrosis factor and interleukin-1α increase vascular endothelial permeability. *Am. J. Physiol.* **257**, L399-L410

Rubin, A.M. & Kang, H. (1987). Cerebral blindness and encephalopathy with cyclosporin A toxicity. *Neurology* **37**, 1072-1076.

Rudge, P., Koetsier, J.C., Mertin, J., Mispelblom Beyer, J.O., van Walbeek, H.K., Clifford Jones, R., Harrison, J., Robinson, K., Mellein, B., Poole, T., Stokvis, J.C.J.M. & Timonen, P. (1989). Randomised double-blind controlled trial of cyclosporin in multiple sclerosis. *J Neurol Neurosurg Psychiatry* **52**, 559-565.

Rumjanek, V.M., Leyton, J. & Morley, J. (1984a). Mononuclear cell accumulation and plasma protein extravasation (PPE) during induction, remission and re-challenge of experimental allergic encephalomyelitis in the rat. *J. Neurolog. Sci.* **65**, 81-92.

Rumjanek, V.M., Smith, L.A. & Morley, J. (1984b). Modulation by cyclosporin-A of mononuclear cell distribution during experimental allergic encehpalomyelitis. *Int. J. Immunopharmac.* **6**, 99-104.

Russo-Marie, F., Paing, M. & Duval, D. (1979). Involvement of glucocorticoid receptors in steroid-induced inhibition of prostaglandin secretion. *J. Biol. Chem.* **254**, 8498-8504.

Ruutiainen, J., Salonen, R., Halonen, P., Panelius, M., Eskola, J. & Salmi, A. (1991). Treatment of acute exacerbations in early multiple sclerosis: cyclosporin A or prednisolone? *Acta Neurol Scand* **83**, 52-54.

Ryffel, B. (1993). Cyclosporin binding proteins. Identification, distribution, function and relation to FK binding proteins. *Biochem Pharmacol* **46**, 1-12.

Sakata, A., Tamai, I., Kawazu, K., Deguchi, Y., Ohnishi, T., Saheki, A. & Tsuji, A. (1994). *In vivo* evidence for ATP-dependent and P-glycoprotein-mediated transport of cyclosporin A at the blood-brain barrier. *Biochem Pharmacol* **48**, 1989-1992.

Scharrer, E. (1944). *Q. Rev. Biol.* **19**, 308

Schauf, C.L., Schauf, V., Davis, F.A. & Mizen, M.R. (1978). Complement-dependent serum: Neuroelectric blocking activity in multiple sclerosis. *Neurology* **28**, 426-430.

Schauf, C.L., Pencek, T.L., Davis, F.A. & Rooney, M.W. (1981). Physiological basis for neuroelectric blocking activity in multiple sclerosis. *Neurology* **31**, 1337-1341.

Schinkel, A.H., Wagenaar, E., van Deemter, L., Mol, C.A.A.M. & Borst, P. (1995). Absence of the mdr1a P-glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin and cyclosporin A. *J. Clin. Invest.* **96**, 1698-1705.

Schultze, G., Stahl, P., Seiber, G., Offerman, G. & Molzahn, M. (1981). Effect of cyclosporine on the production of prostinoids by cultured peripheral lymphocytes. *Transplant. Proc.* **16**, 1214-1217

Schweizer, M. & Richter, C. (1996). Peroxynitrite stimulates the pyridine nucleotide-linked Ca^{2+} release from intact rat liver mitochondria. *Biochemistry.* **35**, 4524-4528.

Scott, G.S., Lees, P., Williams, K.I. & Bolton, C. (1994). The role of nitric oxide (NO) in neurovascular disruption during the development of experimental allergic encephalomyelitis (EAE) in Lewis rats. *J. Physiol.* **480P**, P19.

Scott, G.S., Williams, K,I. & Bolton,C. A pharmacological study on the role of nitric oxide in the pathogenesis of experimental allergic encephalomyelitis. *Inflammation Research.* In press.

Sears, E.S., Tindall, R.S.A. & Zarnow, H. (1978). Active multiple sclerosis. Enhanced computerized tomographic imaging of lesions and the effect of corticosteroids. *Arch Neurol* **35**, 426-434.

Seeldrayers, P.A., Syha, J., Morrissey, S.P., Stodal, H., Vass, K., Jung, S., Gneiting, T., Lassmann, H., Haase, A., Hartung, H. & Toyka, K.V. (1993). Magnetic resonance imaging investigation of blood-brain barrier damage in adoptive transfer experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **46**, 199-206.

Seiler,N. (1983). Analytical methods for amines. *Methods in Enzymology.* **94**, 10-25.

Senjo, M., Ishibashi, T., Terashima, T. & Inoue, Y. (1986). Correlation between astrogliogenesis and blood-brain barrier formation: Immunocytochemistry demonstration by using astroglia-specific enzyme glutathione S-transferase. *Neurosci. Lett.* **66**, 39-42.

Serrano, M.A.R., Curi, R., Parry-Billings, M., Williams, J.F. & Newsholme E.A. (1993). Effects of glucocorticoids on lymphocyte metabolism. *Am. J. Physiol.* **264**, E24-E28.

Shapira, Y., Setton, D., Artru, A.A. & Shohami, E. (1993). Blood-brain barrier permeability, cerebral edema, and neurological function after closed head injury in rats. *Anesth. Analg.* **77**, 141-148.

Sharief, M.K., Noori, M.A., Ciardi, M., Cirelli, A. & Thompson, E.J. (1993). Increased levels of circulating ICAM-1 in serum and cerebrospinal fluid of patients with active multiple sclerosis. Correlation with TNF-α and blood-brain barrier damage. *J. Neuroimmunol.* **43**, 15-22.

Sharma, H.S., Nyberg, F., Cervos-Navarro, J. & Dey, P.K. (1992). Histamine modulates heat stress-induced changes in blood-brain barrier permeability, cerebral blood flow, brain oedema and serotonin levels: an experimental study in conscious young rats. *Neuroscience* **50**, 445-454.

Shaw, C.M. & Alvord, E.C. (1984). A morphologic comparison of three experimental models of experimental allergic encephalomyelitis with multiple sclerosis. In *Experimental allergic encephalomyelitis: A useful model for multiple sclerosis*

(Alvord, E.C., Kies, M.W. and Suckling, A.J. eds.), Alan R. Liss, New York, pp. 61-66.

Shukla, A., Dikshit, M. & Srimal, R.C. (1996). Nitric oxide-dependent blood-brain barrier permeability alteration in the rat brain. *Experientia* **52**, 136-140.

Shukla, A., Shukla, R., Dikshit, M. & Srimal, R.C. (1993). Alterations in free radical scavenging mechanisms following blood-brain barrier disruption. *Free Radical Biol. Med.* **15**, 97-100.

Shulman, H., Striker, G., Deeg, H.J., Kennedy, M., Storb, R. & Thomas, E.D. (1981). Nephrotoxicity of cyclosporin A after allogenic marrow transplantation. *New Engl. J. Med.* **305**, 1392-1395.

Simmons, R.D., Bernard, C.C.A., Singer, G. & Carnegie, P.R. (1982). Experimental autoimmune encephalomyelitis. An anatomically-based explanation of clinical progression in rodents. *J. Neuroimmunol.* **3**, 307-318.

Simmons, R.D., Bernard, C.C.A., Kerlero de Rosbo, N. & Carnegie, P.R. (1984). Criteria for an adequate explanation of typical clinaical signs of EAE in rodents. In *Experimental allergic encephalomyelitis: A useful model for multiple sclerosis* (Alvord, E.C., Kies, M.W. and Suckling, A.J. eds.), Alan R. Liss,Inc. New York, pp. 23-29.

Sisson, W.B. & Oldendorf, W.H. (1971). Brain distribution spaces of mannitol-^{3}H, inulin-^{14}C and dextran ^{14}C in the rat. *Am. J. Physiol.* **221**, 214-217.

Sminia, T., de Groot, C.J.A., Dijkstra, C.D., Koetsier, J.C. & Polman, C.H. (1987). Macrophages in the central nervous system of the rat. *Immunobiol.* **174**, 43-50.

Smith, M.E., Somera, F.P., Saldivar, R., Massacesi, L. & Trotter, J. (1984). DNA changes in the spinal cord of rats with experimental allergic encephalomyelitis. *J. Neurochem.* **43**, 1635-1641.

Smith, M.E. & Eng, L.F. (1988). The development of the gliotic plaque in experimental allergic encephalomyelitis. In *Current issues in neural regeneration research* (Reier, P.J., Bunge, R.P. and Seil, F.J. eds.), Alan R. Liss, New York, pp. 291-300.

Sobel, R.A., Natale, J.M. & Schneeberger, E.E. (1987). The immunopathology of acute experimental allergic encephalomyelitis. IV. An ultrastructural immunocytochemical study of class II major histocompatibility complex molecule (Ia) expression. *J Neuropathol Exp Neurol* **46**, 239-249.

Sobel, R.A., Schneeberger, E.E. & Colvin, R.B. (1988). The immunopathology of acute experimental allergic encephalomyelitis. *Am J Pathol* **131**, 547-558.

Steiner, J.P., Dawson, T.M., Fotuhi, M., Glatt, C.E., Snowman, A.M., Cohen, N. & Snyder, S.H. (1992). High brain densities of the immunophilin FKBP colocalized with calcineurin. *Nature* **358**, 584-587.

Steinman, L. (1992). Multiple sclerosis and its animal models: the role of the major histocompatibility complex and the T cell receptor repertoire. *Springer Semin. Immunopathol.* **14**, 79-93.

Stepkowski, S.M. & Kahan, B.D. (1993). Synergistic activity of the triple combination cyclosporine, rapamycin and brequinar. *Transplant Proc.* **25**, 29-31.

Stewart, P.A. & Wiley, M.J. (1981). Developing nervous tissue induces formation of blood-brain barrier characteristics in invading endothelial cells: A study using quail-chick transplantation chimeras. *Dev. Biol.* **84**, 183-192.

Stone, S.H. (1961). Transfer of allergic encephalomyelitis by lymph node cells in inbred guinea pigs. *Science* **134**, 619-620.

Stubbs, S.S. & Morrell, R.M. (1973). Intravenous methylprednisolone sodium succinate: adverse reactions reported in association with immunosuppressive therapy. *Transplant Proc.* **5**, 1145-6.

Suckling, A.J., Reiber, H., Kirby, J.A. & Rumsby, M.G. (1984). Blood-cerebrospinal fluid barrier permeability changes in guinea pigs inoculated for chronic relapsing experimental allergic encephalomyelitis or with adjuvant alone. In *Experimental Allergic Encephalomyelitis: A Useful Model for Multiple Sclerosis* (Alvord, E.C., Kies, M.W. and Suckling, A.J. eds.), Alan R Liss, Inc. New York pp. 75-80.

Tai, P.K., Albers, M.W., Chang, H., Faber, L.E. & Schreiber, S.L. (1992). Association of a 59-kilodalton immunophilin with the glucocorticoid receptor complex. *Science* **256**, 1315-1318.

Tao-Cheng, J. & Brightman, M.W. (1988). Development of membrane interactions between brain endothelial cells and astrocytes *in vitro*. *Int. J. Dev. Neurosci.* **6**, 25-37.

Thiru, S., Collier, D.St J. & Calne, R. (1987). Pathological studies in canine and baboon renal allograft recipients immunosuppressed with FK-506. *Transplant. Proc.* **19**, 98-99.

Thomson, A.W. & Starzl, T.E. (1993). New immunosuppressive drugs: Mechanistic insights and potential therapeutic advances. *Immunological Reviews* **136**, 71-98.

Tosaki, A., Koltai, M., Joo, F., Adam,G., Szerdahelyi, P., Lepran, I., Takats, I. & Szekeres, L. (1985) Actinomycin-D suppresses the protective effect of dexamethasone in rats affected by global cerebral-ischemia. *Stroke* **16**, 501-505.

Tournier-Lasserve, E. & Bach, J. (1993). The immunogenetics of myasthenia gravis, multiple sclerosis and their animal models. *J. Neuroimmunol.* **47**, 103-114.

Tourtellotte, W.W., Shapshak, P., Baumhefner, R.W., Staugaitis, S.M. & Syndulko, K. (1984). Laboratory aids in the diagnosis of multiple sclerosis (MS). In *Experimental allergic encephalomyelitis: A useful model for multiple sclerosis* (Alvord, E.C., Kies, M.W. and Suckling, A.J. eds.), Alan R. Liss, Inc. New York, pp. 313-321.

Traugott, U., Shevach, E., Chiba, J., Stone, S.H. & Raine, C.S. (1982). Acute experimental autoimmune encephalomyelitis: T- and B-cell distribution within the target organ. *Cell. Immunol.* **70**, 345-356.

Traugott, U., Raine, C.S. & McFarlin, D.E. (1985). Acute experimental allergic encephalomyelitis in the mouse: Immunopathology of the developing lesion. *Cell. Immunol.* **91**, 240-254.

Traugott, U. (1989). Detailed analysis of early immunopathologic events during lesion formation in acute experimental autoimmune encephalomyelitis. *Cell. Immunol.* **119**, 114-129.

Traugott, U., Schevach, E., Chiba, J., Stone, J.H. & Raine, C.S. (1996). Autoimmune encephalomyelitis: simultaneous identification of T and B cells in the target organ. *Science* **214**, 1251-1253.

Tricklebank, M.D., Singh, L., Oles, R.J., Preston, C. & Iversen, S.D. (1989). The behavioural effects of MK-801: a comparison with antagonists acting non-competitively at the NMDA receptor. *Eur. J. Pharmacol.* **167**, 127-135.

Troiano, R., Cook, S.D. & Dowling, P.C. (1987). Steroid therapy in multiple sclerosis. *Arch Neurol* **44**, 803-807.

Trout, J.J., Koenig, H., Goldstone, A.D. & Lu, C.Y. (1986). Blood-brain barrier breakdown by cold injury. Polyamine signals mediate acute stimulation of endocytosis, vesicular transport, and microvillus formation in rat cerebral capillaries. *Lab. Invest.* **55**, 622-631.

Tunkel, A.R. & Scheld, W.M. (1993). Pathogenesis and pathophysiology of bacterial meningitis. *Annu. Rev. Med.* **44**, 103-120.

Turski, L. & Turski, W.A. (1993). Towards an understanding of the role of glutamate in neurodegenerative disorders: energy metabolism and neuropathology. *Experientia* **49**, 1064-1072.

van Deurs, B. (1980). Structural aspects of brain barriers, with special reference to the permeability of the cerebral endothelium and choroidal epithelium. *Int. Rev. Cytol.* **65**, 117-191.

Venkataramanan, R., Warty, V.S., Zemaitis, M.A., Sanghvi, A.T., Burckart, G.J., Seltman, H., Todo, S., Makowka, L. & Starzl, T.E. (1987). Biopharmaceutical aspects of FK506. *Transplant. Proc.* **19**, 30-35.

Villareal, M.S., Klaustermeyer, W.B., Hahn, T.J. & Gordon, E.H. (1996). Osteoporosis in steroid-dependent asthma. *Ann. Allergy Asthma Immunol.* **76**, 369-372.

Villas, P.A., Dronsfield, M.J. & Blankenhorn, E.P. (1991). Experimental allergic encephalomyelitis and corticosterone studies in resistant and susceptible rat strains. *Clin. Immunol. Immunopathol.* **61**, 29-40.

Waage, A. & Bakke, O. (1988). Glucocorticoids suppress the production of tumour necrosis factor by lipopolysaccharide-stimulated human monocytes. *Immunology* **63**, 299-302.

Wang, Q., Yang, H., Miller, D.W. & Elmquist, W.F. (1995). Effect of the P-glycoprotein inhibitor, cyclosporin A, on the distribution of rhodamine-123 to the brain: an in vivo microdialysis study in freely moving rats. *Biochem. Biophys. Res. Comm.* **211**, 719-726.

Weber, F., Meyermann, R. & Hempel, K. (1991). Experimental allergic encephalomyelitis - prophylactic and therapeutic treatment with the cyclooxygenase inhibitor piroxicam (Feldene). *Int. Arch Allergy Appl. Immunol.* **95**, 136-141.

Weidenfeld, J., Lysy, J. & Shohami, E. (1987). Effect of dexamethasone on prostaglandin synthesis in various areas of the rat brain. *J. Neurochem.* **48**, 1351-1354.

Weiner, H.L. & Paty, D.W. (1989). Diagnostic and therapeutic trials in multiple sclerosis: A new look. *Neurology* **39**, 972-976.

Wekerle, H., Linington, C., Lassmann, H. & Meyermann, R. (1986). Cellular immune reactivity within the CNS. *TINS* **9**, 271-277.

Wesselmann, U., Konkol, R.J., Leo, G.L., Roerig, D.L. & Harder, D.R. (1987). Altered splenic catecholamine concentrations during experimental allergic encephalomyelitis. *Pharmacol. Biochem. Behav.* **26**, 851-854.

Whisler, R.L., Lindsey, J.A., Proctor, K.V.W., Morisaki, N. & Cornwell, D.G. (1984). Characteristics of cyclosporine induction of increased prostaglandin levels from human peripheral blood monocytes. *Transplantation* **38**, 377-381.

Wilcox, C.E., Baker, D., Butter, C., Willoughby, D.A. & Turk, J.L. (1989). Differential expression of guinea pig class II major histocompatibility complex antigens on vascular endothelial cells *in vitro* and in experimental allergic encephalomyelitis. *Cell. Immunol.* **120**, 82-91.

Wilcox, C.E., Ward, A.M.V., Evans, A., Baker, D., Rothlein, R. & Turk, J.L. (1990). Endothelial cell expression of the intercellular adhesion molecule-1 (ICAM-1) in the central nervous system of guinea pigs during acute and chronic relapsing experimental allergic encephalomyelitis. *J. Neuroimmunol.* **30**, 43-51.

Wiley, C.A., Johnson, R.T. & Reingold, S.C. (1992). Neurological consequences of immune dysfunction: Lessons from HIV infection and multiple sclerosis. *J. Neuroimmunol.* **40**, 115-120.

Williams, I.A., Baylis, E.M. & Shipley, M.E. (1982). A double-blind placebo-controlled trial of methylprednisolone pulse therapy in active rheumatoid disease. *Lancet* **ii**, 237-40.

Willoughby, E.W., Grochowski, E., Li, D.K.B., Oger, J., Kastrukoff, L.F. & Paty, D.W. (1989). Serial magnetic resonance scanning in multiple sclerosis: a second prospective study in relapsing patients. *Ann. Neurol.* **25**, 43-49.

Woerly, G., Weber, E. & Ryffel, B. (1994). Demonstration of ternary immunophilin-calcineurin complexes with the immunosuppressants cyclosporin and macrolide FK506. *Biochem Pharmacol* **47**, 1435-1443.

Wolff, J.E.A., Laterra, J. & Goldstein, G.W. (1992). Steroid inhibition of neural microvessel morphogenesis *in vitro*: receptor mediation and astroglial dependence. *J. Neurochem.* **58**, 1023-1032.

Yamaguchi, M., Shirakata, S., Yamasaki, S. & Matsumoto, S. (1976). Ischemic brain edema and compression brain edema. Water content, blood-brain barrier and circulation. *Stroke* **7**, 77-83.

Zeevalk, G.D., Nicklas, W.J. & Sonsalla, P.K. (1994). NMDA receptor involvement in two animal models of Parkinson's disease. *Neurobiol. Aging* **15**, 269-270.

Zeevi, A., Duquesnoy, R., Eiras, G., Rabinowich, H., Todo, S., Makowka, L. & Starzl, T.E. (1987). Immunosuppressive effect of FK506 on in vitro lymphocyte alloactivation: synergism with cyclosporin A. *Transplant. Proc.* **19**, 40-44.

Zhao, W., Tilton, R.G., Corbett, J.A., McDaniel, M.L., Misko, T.P., Williamson, J.R., Cross, A.H. & Hickey, W.F. (1996). Experimental allergic encephalomyelitis in the rat is inhibited by aminoguanidine, an inhibitor of nitric oxide synthase. *J. Neuroimmunol.* **64**, 123-133.

Ziylan, Y.Z., LeFauconnier, J.M., Bernard, G. & Bourre, J.M. (1988). Effect of dexamethasone on transport of α-aminoisobutyric acid and sucrose across the blood-brain barrier. *J. Neurochem.* **51**, 1338-1342.

Zuccarello, M. & Anderson, D.K. (1989). Protective effect of a 21-aminosteroid on the blood-brain barrier following subarachnoid hemorrage in rats. *Stroke* **20**, 367-371.

Appendix

Physical data for Radionuclides

Hydrogen - 3 (Tritium)
The atom undergoes beta minus decay with a half life of 12.28 years.

$$^{3}_{1}H \rightarrow {}^{3}_{2}He + \beta \qquad t_{\frac{1}{2}} = 12.28 \text{ years}$$

Maximum β Energy = 0.019MeV (100%)
Energy emission = 0.019MeV

Iodine - 125
The atom undergoes electron capture decay with a half life of 60 days.

$$^{125}_{53}I \rightarrow {}^{125}_{52}Te + \gamma \qquad t_{\frac{1}{2}} = 60 \text{ days}$$

Principle Radiation Emissions:
Gamma = 0.035MeV (6.5%)
Kα X-rays = 0.027MeV (112.5%)
Kβ X-rays = 0.031MeV (25.4%)
Energy Emission = 0.177MeV

Indium - 111
The atom undergoes electron capture decay with a half life of 67 hours.

$$^{111}_{49}In \rightarrow {}^{111}_{48}Cd + \gamma \qquad t_{\frac{1}{2}} = 67 \text{ hours}$$

Principle Radiation Emissions:
Gamma = 0.171MeV (90%)
Gamma = 0.245MeV (94%)
Energy Emission = 0.850MeV

Indium -113m
The atom undergoes isomeric level decay with a half life of 100 minutes.

$$^{113m}_{49}In \rightarrow {}^{113}_{49}In + \gamma \qquad t_{\frac{1}{2}} = 100 \text{ minutes}$$

Principle Radiation Emission:
Gamma = 0.393MeV(100%)
Energy Emission = 0.393MeV

Pergamon

Int. J. Immunopharmac., Vol. 17, No. 6, pp. 497–503, 1995
Elsevier Science Ltd

0192–0561/95 $9.50 + .00

0192–0561(95)00034-8

INHIBITION OF BLOOD–BRAIN BARRIER DISRUPTION IN EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS BY SHORT-TERM THERAPY WITH DEXAMETHASONE OR CYCLOSPORIN A

C. PAUL and C. BOLTON

Pharmacology Group, School of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, U.K.

(*Received* 13 *May* 1994 *and in final form* 29 *June* 1994)

Abstract — Double radioisotope measurement of neurovascular integrity in Lewis rats inoculated for experimental allergic encephalomyelitis (EAE) showed abnormal elevation of albumin extravasation in the cerebellum, medulla–pons and cervical spinal cord at the time of clinical manifestation. Therapeutically administered dexamethasone (Dex) (0.1–1 mg/kg body weight) or cyclosporin A (CsA) (25–75 mg/kg body weight) dose-dependently reduced albumin movement across the blood–brain barrier (BBB). Dex at a dose of 1 mg/kg completely suppressed abnormal BBB permeability in all tissues ($P \leq 0.001$), while CsA at the highest dose of 75 mg/kg achieved highly significant ($P \leq 0.001$), but not complete, suppression of aberrant barrier leakage in the areas studied. The implications of these findings to possible drug action at the immunocompromised cerebrovasculature are discussed.

Keywords: blood–brain barrier, experimental allergic encephalomyelitis, dexamethasone, cyclosporin A.

A fundamental stage in the onset of multiple sclerosis (MS) and the animal counterpart, experimental allergic encephalomyelitis (EAE), is the breakdown of normal blood–brain barrier (BBB) function. Principally, the BBB is composed of a continuous endothelial cell layer, in close association with astrocytic processes on the abluminal surface. The endothelial cells of the cerebral vasculature possess a number of unique properties, which include intercellular tight junctions, a reduced number of pinocytotic vesicles, and a high electrical resistance (Risau & Wolburg, 1990). These special characteristics aid the enhanced selectivity of the barrier, allowing movement of only vital nutrients into the central nervous system (CNS), often by employing active transport mechanisms (Bradbury, 1985; Goldstein & Betz, 1986). Consequently, exposure of neuronal tissue to vascular neurotoxins, ion fluxes or other deleterious systemic factors is prevented and homeostasis within the cerebral environment is maintained.

Barrier dysfunction in MS leads to oedema formation and inflammatory cell infiltration, which ultimately results in axon demyelination and gliotic scarring of the lesion (Adams, 1977). With the advent of magnetic resonance imaging it has become possible to locate areas where the barrier has been breached by visualizing MS lesions during growth and decline (Grossman *et al.*, 1988; Bastianello *et al.*, 1990). Study of BBB disruption due to neuroimmune events has also been possible during the development of EAE which presents a similar pattern of lesion pathology to MS (Paterson, 1978). Initial work used the vascular tracer typan blue to visualize the abnormally permeable neurovasculature in EAE (Barlow, 1956) and later studies employing radiolabelled compounds quantitated the unregulated entry of plasma constituents into the CNS (Leibowitz, 1969; Leibowitz & Kennedy, 1972; Juhler *et al.*, 1984).

High-dose steroid therapy for the management of acute MS relapses has been shown to lessen the severity of clinical disease and reduce lesion activity by undefined mechanisms (Troiano *et al.*, 1987; Milanese *et al.*, 1989). The glucocorticoids are known to facilitate water movement out of oedematous sites, and scans using gadolinium-DTPA magnetic resonance imaging have recently shown reduced lesion enhancement in MS patients following steroid treatment suggesting drug effects at neurovascular sites (Kesselring *et al.*, 1989; Barkhof *et al.*, 1991; Burnham *et al.*, 1991). Similarly, trials with cyclosporin A (CsA) in MS have indicated a modest clinical effect at high doses, but the drug's

therapeutic ability to modify BBB function has not been assessed (Rudge *et al.*, 1989; The Multiple Sclerosis Study Group, 1990; Ruutiainen *et al.*, 1991).

Work by us and others, employing dexamethasone (Dex) and CsA in either prophylactic or therapeutic dosing regimes, has shown the dramatic curtailment of symptom onset or progression of EAE, (Komarek & Dietrich, 1971; Levine & Sowinski, 1980; Bolton *et al.*, 1982a,b; Hinrichs *et al.*, 1983; Bolton & Flower, 1989; Desai & Barton, 1989). The beneficial effects of Dex and CsA treatment on the course of EAE are thought to arise from a down-regulation of the immune response to encephalitogen. However, Dex and CsA may also influence the development of EAE by direct action on the BBB as several studies have shown the drugs or their degraded products accumulate within or pass across the neurovasculature and provide control over abnormal permeability changes (Pardridge & Mietus, 1979; Nooter *et al.*, 1984; Long & Holaday, 1985; Begley *et al.*, 1990).

As impairment of cerebrovascular integrity is pivotal in the pathogenesis of MS and EAE, limiting BBB dysfunction, whether short-term or for an extended period, may lessen the damage of target tissues and reduce neurological deficits. The aim of the present study was to investigate the ability of Dex and CsA to inhibit abnormal CNS blood vessel permeability during early neurological EAE. The results of the investigation are considered in context with proposed mechanisms of neuroendothelial disruption.

EXPERIMENTAL PROCEDURES

Animals

Inbred male Lewis rats, weighing 200–250 g, were selected from stock bred on site, with food (SDS, diet C.R.M.) and water available *ad libitum*.

Inoculation for EAE

Animals were inoculated for EAE as previously described (Bolton & Flower, 1989). Briefly, an encephalitogenic emulsion, consisting of equal parts of guinea-pig spinal cord, sterile phosphate-buffered saline (PBS), and incomplete Freund's adjuvant was prepared and supplemented with 10 mg/ml *Mycobacterium tuberculosis* H_{37}Ra (Difco Laboratories). Each rat received 0.1 ml of inoculum per hind footpad. Control animals received inoculum without spinal tissue. A minimum of five animals were used per treatment group.

Clinical assessment of EAE

Animal body weight was monitored daily throughout the course of study, beginning day 0 post-inoculation (p.i.). Neurological signs were scored daily subsequent to observed weight loss, as follows: 1 = flaccid tail; 2 = ataxic gait; 3 = partial paralysis of hindlimbs; 4 = complete hindlimb paralysis with associated incontinence.

Drug preparation and administration

Dexamethasone sodium phosphate (4 mg/ml, DBL, U.K.) was diluted in sterile PBS and administered to EAE-sensitised animals subcutaneously twice daily for 2 days from weight loss. Doses for steroid treatment were selected from previous studies (Bolton & Flower, 1989), and ranged from 0.01 to 1 mg/kg body weight. Control sensitized animals received equivalent volumes of sterile PBS.

CsA (Sandoz Laboratories, Basle, Switzerland) was dissolved in olive oil at 25 mg/ml and single oral doses were given to EAE-inoculated rats, commencing on the day of weight loss for 2 days. Treatments were based on earlier work (Bolton *et al.*, 1982b) and ranged from 25 to 75 mg/kg body weight. Control sensitized animals received vehicle alone.

^{111}In-*tropolonate labelling of red blood cells (RBC)*

The method was developed from the studies of Osman & Danpure (1987). Blood from adult Wistar rats was collected by cardiac puncture in citrate–phosphate–dextrose (Sigma), and centrifuged at 300 *g* for 10 min. The plasma/white blood cell layer was collected and respun at 110 *g* for 15 min to generate cell-free plasma. The RBC layer was resuspended in Hepes saline buffer (Gibco) and washed three times at 300 *g* to remove leukocytes, resuspending at a final concentration of 5×10^8 cells/ml. An addition was made of 100 µl ^{111}In-tropolonate [4×10^{-3} M tropolone containing 20 µCi ^{111}In (NEN) per ml for every 5×10^8 RBC, followed by incubation of the mixture at 37° for 20 min. The labelled cells were washed and resuspended at 5×10^9 cells/0.5 ml cell-free plasma for *in vivo* use.

Quantitation of BBB permeability

The permeability of the cerebral vasculature was determined for specific CNS tissues chosen on the basis of previous histological analysis to reflect areas of high and minimal transendothelial inflammatory cell infiltration of parenchymal tissue (Bolton *et al.*, 1984). The extravasation of rat serum albumin (RSA) (Sigma) into

the defined neurological sites was quantitated using a variation of the double radioisotope technique first described by Leibowitz & Kennedy (1972). Briefly, rats received intravenously (i.v.) 10 μCi ^{125}I-RSA (prepared by a modification of the Chloramine-T method described by McConahey & Dixon, 1980) under halothane/O_2 anaesthetic, and 24 h later 5×10^9 ^{111}In-RBC. After 4.5 min circulation of the second isotope cardiac blood was collected into heparin-coated tubes, immediately followed by a lethal injection of euthatal (RMB Animal Health Ltd). Cerebellum, medulla–pons, and cervical spinal tissues were dissected, weighed and the amount of ^{111}In radioactivity recorded on the open channel of a LKB minigamma counter (settings = 100–450 kev). Blood aliquots (100 μl) were counted simultaneously. Samples were stored at –20°C and recounted 3 weeks later on the ^{125}I channel (settings = 20–80 kev) following ^{111}In decay. The individual isotope counts for each tissue were expressed in arbitrary units termed "blood equivalents" (BE) [equation(1)].

$$\frac{\text{cpm / g Tissue}}{\text{cpm / ml Blood}} \times 100 = \text{BE}. \qquad (1)$$

The extravascular blood equivalent (EVBE), a measure of the radiolabelled albumin which has crossed the BBB and accumulated within the CNS, was calculated by equation (2):

$$^{125}\text{I BE} - {}^{111}\text{In BE} = \text{EVBE}. \qquad (2)$$

Statistics

Results were analysed using the Mann–Whitney *U*-test for non-parametric data.

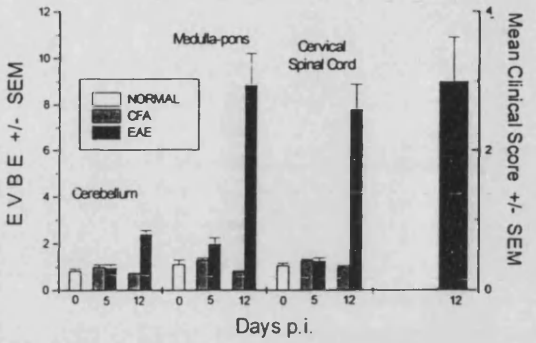


Fig. 1. Profile of protein extravasation (EVBE ± S.E.M.) for the cerebellum, medulla–pons and cervical spinal cord tissues from normal, CFA-inoculated and EAE-sensitized Lewis rats, during pre-neurological and acute phases of EAE.

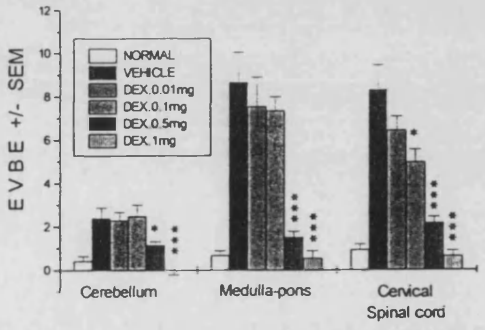


Fig. 2. The ability of Dex treatments (0.01–1.0 mg/kg body weight) to suppress increased BBB permeability in EAE-sensitized Lewis rats (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

RESULTS

Protein extravasation during EAE

Prior to determining the influence of Dex and CsA on cerebrovascular permeability a profile of protein extravasation was established during the development of acute EAE (Fig. 1). Levels of radiolabelled albumin traversing the BBB in normal and control animals were minimal (cerebellum 0.83 ± 0.09; medulla–pons 1.11 ± 0.19; cervical spinal cord 1.06 ± 0.09). Similarly, movement of protein across the neurovasculature in EAE-inoculated animals, 5 days p.i., remained within normal limits. The development of clinical EAE, 12 days p.i., coincided with a significant rise in EVBE values in all tissues (cerebellum 2.39 ± 0.16; medulla–pons 8.84 ± 1.36; cervical spinal cord 7.78 ± 1.10: $P \leq 0.001$). However, the increase in EVBE was not uniform throughout the CNS, with medulla–pons and cervical spinal tissues developing highly permeable barriers while vascular changes in the cerebellum were consistently lower.

The effects of therapeutic Dex administration on BBB permeability

The ability of several therapeutically administered Dex concentrations to inhibit BBB disruption (Fig. 2) and suppress EAE symptoms (Table 1) was assessed by the dual radioisotope technique. Rats dosed with vehicle alone maintained the profile of BBB breakdown characteristic of untreated EAE animals at day 12 p.i. Low-dose Dex (0.01 mg/kg body weight) had no significant effect on suppressing barrier breakdown or modifying the course of EAE. Steroid administration

Table 1. Incidence of neurological symptoms in EAE-sensitized Lewis rats following therapeutic administration of Dex or CsA

Treatment (mg/kg body weight)	$n^{\dagger}$	Mean clinical score ± S.E.M.
Dex		
Vehicle	18	2.73 ± 0.30
0.01	5	3.50 ± 0.50
0.1	11	1.63 ± 0.32*
0.5	7	0.14 ± 0.14**
1	7	0**
CsA		
Vehicle	16	1.91 ± 0.22
25	5	1.20 ± 0.72*
50	7	0.21 ± 0.15**
75	6	0**

†n, Number of rats per treatment.
Significance of difference from vehicle-treated group: *$P \leq 0.05$; **$P \leq 0.001$.

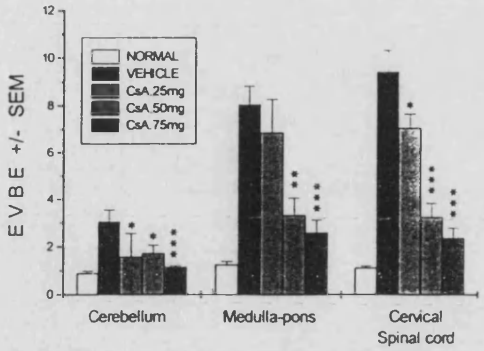


Fig. 3. The ability of CsA treatments (25–75 mg/kg body weight) to suppress increased BBB permeability in EAE-sensitized Lewis rats (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

at 0.1 mg/kg body weight exclusively restricted the movement of radiolabelled albumin in spinal tissues ($P \leq 0.05$) and suppressed the development of neurological signs ($P \leq 0.05$) while other CNS areas remained unaffected. High concentrations of Dex (0.5 and 1.0 mg/kg body weight) dose-dependently inhibited barrier dysfunction in all tissues studied ($P \leq 0.001$) and prevented the occurrence of neurological deficits ($P \leq 0.001$).

The effects of therapeutic CsA administration on BBB permeability

A range of CsA concentrations was employed therapeutically to modify BBB function (Fig. 3) and suppress clinical deficits (Table 1). Vehicle control animals showed a highly disturbed neurovasculature correlating well with untreated time-matched EAE rats. Application of low-dose CsA (25 mg/kg body weight) had a limited but significant inhibitory effect on abnormal neurovascular permeability within the cerebellum and cervical spinal cord ($P \leq 0.05$) and partially suppressed the appearance of symptoms ($P \leq 0.05$). CsA administered at 50 mg/kg body weight restricted loss of BBB integrity in all tissues (cerebellum $P \leq 0.05$; medulla–pons $P \leq 0.01$; cervical spinal cord $P \leq 0.001$) and displayed strong clinical suppression ($P \leq 0.001$). High-dose CsA therapy (75 mg/kg body weight) significantly inhibited aberrant BBB permeability ($P \leq 0.001$) and abolished neurological signs ($P \leq 0.001$) but, in contrast to steroid treatment, did not completely prevent increased protein movement across the cerebrovasculature.

DISCUSSION

The current study has reconfirmed the pattern of BBB breakdown following neuroantigenic challenge, as initially reported by Leibowitz & Kennedy (1972). Also, the heightened level of radiolabelled albumin accumulation within the spinal cord compared to the cerebellum reflects the earlier observation of Rumjanek *et al.* (1984) who found a similar distinction between spinal and brain tissues when examining cellular accumulation within the CNS. In particular, we have employed the dual radioisotope technique to examine the inhibitory action of Dex and CsA on enhanced cerebrovascular permeability during the onset of neurological EAE, and our findings show that both drugs dose-dependently suppress BBB leakage and concomitantly curtail the development of paralytic disease. However, the ability to inhibit atypical neurovascular permeability did differ between the drugs, with only Dex completely counteracting aberrant barrier leakage.

EAE is a complex cell-mediated neurological condition, and pharmacological control over BBB breakdown and the sustained dysfunction of the neuroendothelium may be exerted at various stages in the manifestation of the inflammatory response to encephalitogen. Prophylactic administration of Dex or CsA to EAE-inoculated animals completely inhibits the neurological signs of disease and the collection of inflammatory cells on the abluminal side of the cerebroendothelium by preventing the initial sensitization to inoculum (Komarek & Dietrich, 1971; Bolton *et al.*, 1982a, b; Hinrichs *et al.*, 1983; Bolton & Flower, 1989; Desai & Barton, 1989). Encephalitogenically sensitized immune cells are known to penetrate the BBB and enter the CNS parenchyma before the emergence of clinical EAE and would therefore be beyond the control of a

therapeutic regime as used in the present study (Daniel *et al.*, 1981; Traugott, 1989). However, treatment with Dex or CsA at disease onset may restrict further passage of immunocompetent cells across the neurovasculature by interfering with encephalitogenic priming of lymphoid cells, or by suppressing the release of inflammatory mediators capable of disrupting the cerebral endothelium (Bolton *et al.*, 1986; Dupont, 1988; Lew *et al.*, 1988; Waage & Bakke, 1988). Alternatively, the drugs may counteract abnormal increases in neuroendothelial permeability by direct action on the vasculature. Indeed, the effects of CsA *in vivo* cannot be solely accounted for by the inhibition of T-cell proliferation (Klaus & Chisholm, 1986). Furthermore, several workers have described the *in vivo* and *in vitro* accumulation of CsA within intact and isolated neuroendothelium from where the compound would have the potential to influence endothelial cell function (Nooter *et al.*, 1984; Begley *et al.*, 1990). Similarly, Dex readily crosses the BBB and would be capable of interacting with vessel constituents to modify an immunocompromised barrier (Hedley-Whyte & Hsu, 1986). Studies by Long & Holaday (1985) in normal rats provide strong evidence that endogenous steroids modulate the permeability of the microvasculature as adrenalectomy caused a significant increase in protein extravasation into CNS tissues which could be prevented by glucocorticoid administration.

The mechanisms through which Dex and CsA may exert their suppressive effects on the neurovasculature during early EAE are unclear but are the subject of our ongoing studies. Drugs taken up by the neuroendothelium would be available to modify intracellular processes which ultimately influence the continuity of the BBB. Indeed, Dex and CsA-sensitive cerebroendothelial-dependent pathways, which result in the formation of permeability-inducing factors, have been identified. In particular, the drugs can limit receptor-dependent, enzyme-mediated polyamine production which has been closely linked to profound barrier disruption in non-immune models of neurovascular breakdown through gross physiological changes equivalent to those observed in the cerebroendothelium of animals with EAE (Trout *et al.*, 1986; Claudio *et al.*, 1989; Koenig *et al.*, 1989, 1992; Ryffel, 1993). Comparable receptor-dependent events, inhibitable by Dex and CsA, are also responsible for the generation of the potent vasodilator nitric oxide which has been implicated in the early pathogenesis of EAE and together with the polyamines could exert profound disruptive effects on the cerebral vasculature (MacMicking *et al.*, 1992; Moncada, 1992; Palmer *et al.*, 1992; Moncada & Higgs, 1993; Ludowyk *et al.*, 1993; Mühl *et al.*, 1993). Interestingly, preliminary investigations by us have revealed the polyamine and nitric oxide content of CNS-derived vessels to be raised at the onset of EAE, thus supporting an inductive role for these compounds in loss of BBB integrity (Bolton *et al.*, 1994).

In conclusion, this investigation has determined the relative efficacies of Dex and CsA to suppress enhanced BBB permeability during early neurological EAE in the Lewis rat. The use of a short therapeutic dosing regime to limit neurovascular damage has allowed consideration of the drugs' influence on the immunocompromised BBB in addition to their interruptive effects on neuroimmune cell generation. Studying the direct effects of Dex and CsA on the cerebroendothelium during EAE may provide a specific and relevant site to target in attempting to control the central inflammatory events characteristic of the experimental and human demyelinating diseases.

Acknowledgements — We wish to thank Mrs Lesley Moore for her technical assistance, Sandoz Laboratories, Switzerland, for their kind gift of cyclosporin A and the Multiple Sclerosis Society of Great Britain and Northern Ireland for financial support.

REFERENCES

Adams, C. W. M. (1977). Pathology of multiple sclerosis: progression of the lesion. *Br. med. Bull.*, **33**, 15–20.

Barkhof, F., Hommes, O. R., Scheltens, P. & Valk, J. (1991). Quantitative MRI changes in gadolinium-DTPA enhancement after high-dose intravenous methylprednisolone in multiple sclerosis. *Neurology*, **41**, 1219–1222.

Barlow, C. F. (1956). A study of abnormal blood–brain barrier permeability in experimental allergic encephalomyelitis. *J. Neuropath. exp. Neurol.*, **15**, 196–208.

Bastianello, S., Pozzilli, C., Bernardi, S., Bozzao, L., Fantozzi, L. M., Buttinelli, C. & Fieschi, C. (1990). Serial study of gadolinium-DTPA MRI enhancement in multiple sclerosis. *Neurology*, **40**, 591–595.

Begley, D. J., Squires, L. K., Zlokovic, B. W., Mitrovic, D. M., Hughes, C. C. W., Revest, P. A. & Greenwood, J. (1990). Permeability of the blood–brain barrier to the imunosuppressive cyclic peptide cyclosporin A. *J. Neurochem.*, **55**, 1222–1230.

Bolton, C., Allsopp, G. & Cuzner, M. L. (1982a). The effect of cyclosporin A on adoptive transfer of experimental allergic encephalomyelitis in the Lewis rat. *Clin. exp. Immun.*, **47**, 127–132.

Bolton, C., Borel, J. F., Cuzner, M. L., Davison, A. N. & Turner, A. M. (1982b). Immunosuppression by cyclosporin A of experimental allergic encephalomyelitis. *J. Neurol. Sci.*, **56**, 147–153.

Bolton, C. & Flower, R. J. (1989). The effects of the anti-glucocorticoid RU 38486 on steroid-mediated suppression of experimental allergic encephalomyelitis (EAE) in the Lewis rat. *Life Sci.*, **45**, 97–104.

Bolton, C., Gordon, D. & Turk, J. L. (1984). Prostaglandin and thromboxane levels in central nervous system tissues from rats during the induction and development of experimental allergic encephalomyelitis (EAE). *Immunopharmacology*, **7**, 101–107.

Bolton, C., Lees, P., Paul, C., Scott, G. S., Williams, K. I. & Woodyer, P. (1994). Aspects of the biochemical pharmacology of neurovascular disruption in experimental allergic encephalomyelitis (EAE). *J. Neuroimmun.*, **52**, 113.

Bolton, C., Parker, D., McLeod, J. & Turk, J. L. (1986). A study of the prostaglandin and thromboxane content of the central nervous tissues with the development of chronic relapsing allergic encephalomyelitis. *J. Neuroimmun.*, **10**, 201–208.

Bradbury, M. W. B. (1985). The blood–brain barrier: transport across the cerebral endothelium. *Circulation Res.*, **57**, 213–222.

Burnham, J. A., Wright, R. R., Driesbach, J. & Murray, R. S. (1991). The effect of high-dose steroids on MRI gadolinium enhancement in acute demyelinating lesions. *Neurology*, **41**, 1349–1354.

Claudio, L., Kress, Y., Norton, W. T. & Brosnan, C. F. (1989). Increased vesicular transport and decreased mitochondrial content in blood–brain barrier endothelial cells during experimental autoimmune encephalomyelitis. *Am. J. Path.*, **135**, 1157–1168.

Daniel, P. M., Lam, D. K. C. & Pratt, O. E. (1981). Changes in the effectiveness of the blood–brain and blood–spinal cord barriers in experimental allergic encephalomyelitis. *J. Neurol. Sci.*, **52**, 211–219.

Desai, S. & Barton, R. (1989). Pharmacological comparison of active and passive experimental allergic encephalomyelitis in the rat. *Agents. Actions*, **27**, 351–355.

Dupont, E. (1988). Immunological actions of corticosteroids and cyclosporin A. *Curr. Opin. Immun.*, **1**, 253–256.

Goldstein, G. W. & Betz, A. L. (1986). The blood–brain barrier. *Scient. Am.*, **255**, 70–79.

Grossman, R. I., Braffman, B. H., Brorson, J. R., Goldberg, H. I., Silberberg, D. H. & Gonzalez-Scarano, F. (1988). Multiple sclerosis: serial study of gadolinium-enhanced MR imaging. *Radiology*, **169**, 117–122.

Hedley-Whyte, E. T. & Hsu, D. W. (1986). Effect of dexamethasone on blood–brain barrier in the normal mouse. *Ann. Neurol.*, **19**, 373–377.

Hinrichs, D. J., Wegmann, K. W. & Peters, B. A. (1983). The influence of cyclosporin A on the development of actively induced and passively transferred experimental allergic encephalomyelitis. *Cell. Immun.*, **77**, 202–209.

Juhler, M., Barry, D. I., Offner, H., Konat, G., Klinken, L. & Paulson, O. B. (1984). Blood–brain and blood–spinal cord barrier permeability during the course of experimental allergic encephalomyelitis in the rat. *Brain Res.*, **302**, 347–355.

Kesselring, J., Miller, D. H., Macmanus, D. G., Johnson, G., Milligan, N. M., Scolding, N., Compston, D. A. S. & McDonald, W. I. (1989). Quantitative magnetic resonance imaging in multiple sclerosis: the effect of high dose intravenous methylprednisolone. *J. Neurol. Neurosurg. Psychiat.*, **52**, 14–17.

Klaus, G. G. B. & Chisholm, P. M. (1986). Does cyclosporin act *in vivo* as it does *in vitro? Immun. Today*, **7**, 101–103.

Koenig, H., Goldstone, A. D. & Lu, C. Y. (1989). Blood–brain barrier breakdown in cold-injured brain is linked to a biphasic stimulation of ornithine decarboxylase activity and polyamine synthesis: both are coordinately inhibited by verapamil, dexamethasone, and aspirin. *J. Neurochem.*, **52**, 101–109.

Koenig, H., Trout, J. J., Goldstone, A. D. & Lu, C. Y. (1992). Capillary NMDA receptors regulate blood–brain barrier function and breakdown. *Brain Res.*, **588**, 297–303.

Komarek, A. & Dietrich, F. M. (1971). Chemical prevention of experimental allergic encephalomyelitis in rats: a quantitative evaluation of steroids and various non-steroid drugs. *Archs int. Pharmacodyn.*, **193**, 249–257.

Leibowitz, S. (1969). Cerebral vascular permeability in experimental allergic encephalomyelitis. *Neuropath. Pol.*, **7**, 303–309.

Leibowitz, S. & Kennedy, L. (1972). Cerebral vascular permeability and cellular infiltration in experimental allergic encephalomyelitis. *Immunology*, **22**, 859–869.

Levine, S. & Sowinski, R. (1980). Therapy of allergic encephalomyelitis in rats after onset of paralysis. In *The Suppression of Experimental Allergic Encephalomyelitis and Multiple Sclerosis* (eds Davison, A. N. and Cuzner, M. L.), pp. 199–209. Academic Press, London.

Lew, W., Oppenheim, J. J. & Matsushima, K. (1988). Analysis of the suppression of IL-1α and IL-1β production in human peripheral blood mononuclear adherent cells by a glucocorticoid hormone. *J. Immun.*, **140**, 1895–1902.

Long, J. B. & Holaday, J. W. (1985). Blood–brain barrier: endogenous modulation by adrenal-cortical function. *Science*, **227**, 1580–1582.

Ludowyk, P. A., Hughes, W., Hugh, A., Willenborg, D. O., Rockett, K. A. & Parish, C. R. (1993). Astrocytic hypertrophy: an important pathological feature of chronic experimental autoimmune encephalitis in aged rats. *J. Neuroimmun.*, **48**, 121–134.

MacMicking, J. D., Willenborg, D. O., Weidemann, M. J., Rockett, K. A. & Cowden, W. B. (1992). Elevated secretion of reactive nitrogen and oxygen intermediates by inflammatory leukocytes in hyperacute experimental autoimmune encephalomyelitis: enhancement by the soluble products of encephalitogenic T-cells. *J. exp. Med.*, **176**, 303–307.

McConahey, P. J. & Dixon, F. J. (1980). Radioiodination of proteins by the use of the Chloamine-T method. In *Methods in Enzymology*, Vol. 70 (eds van Vunakis, H. and Langone, J. J.), pp. 210–213. Academic Press, London.

Milanese, C., La Mantia, L., Salmaggi, A., Campi, A., Eoli, M., Scaioli, V., Nespolo, A. & Corridori, F. (1989). Double-blind randomized trial of ACTH versus dexamethasone versus methylprednisolone in multiple sclerosis bouts. *Eur. Neurol.*, **29**, 10–14.

Moncada, S. (1992). The 1991 Ulf von Euler lecture. The L-arginine : nitric oxide pathway. *Acta Physiol. scand.*, **145**, 201–227.

Moncada, S. & Higgs, A. (1993). The L-arginine-nitric oxide pathway. *N. Engl. J. Med.*, **329**, 2002–2012.

Mühl, H., Kunz, D., Rob, P. & Pfeilschifter, J. (1993). Cyclosporin derivatives inhibit interleukin-1β induction of nitric oxide synthase in renal mesangial cells. *Eur. J. Pharmac.*, **249**, 95–100.

Nooter, K., Meershoek, B., Spaans, W., Sonneveld, P., Oostrum, R. & Deurloo, J. (1984). Blood and tissue distribution of cyclosporin A after a single oral dose in the rat. *Experientia*, **40**, 559–561.

Osman, S. & Danpure, H. J. (1987). A simple *in vitro* method of radiolabelling human erythrocytes in whole blood with ^{113m}In-tropolanate. *Eur. J. Haematol.*, **39**, 125–127.

Palmer, R. M. J., Bridge, L., Foxwell, N. A. & Moncada, S. (1992). The role of nitric oxide in endothelial cell damage and its inhibition by glucocorticoids. *Br. J. Pharmac.*, **105**, 11–12.

Pardridge, W. M. & Mietus, L. J. (1979). Transport of steroid hormones through the rat blood–brain barrier: primary role of albumin-bound hormone. *J. clin. Invest.*, **64**, 145–154.

Paterson, P. Y. (1978). The demyelinating diseases: clinical and experimental studies in animals and man. In *Immunological Diseases* (eds Samter, M., Alexander, N., Rose, B., Sherman, W. B., Talmage, D. W. and Vaughn, J. H.), pp. 1400–1435. Little Brown, Boston.

Risau, W. & Wolburg, H. (1990). Development of the blood–brain barrier. *Trends Neurosci.*, **13**, 174–178.

Rudge, P., Koetsier, J. C., Mertin, J., Mispelblom Beyer, J. O., Van Walbeek, H. K., Clifford Jones, R., Harrison, J., Robinson, K., Mellein, B., Poole, T., Stokvis, J. C. J. M. & Timosen, P. (1989). Randomised double-blind controlled trial of cyclosporin in multiple sclerosis. *J. Neurol. Neurosurg. Psychiat.*, **52**, 559–565.

Rumjanek, V. M., Leyton, J. & Morley, J. (1984). Mononuclear cell accumulation and plasma protein extravasation (PPE) during induction, remission and re-challenge of experimental allergic encephalomyelitis in the rat. *J. Neurol. Sci.*, **65**, 81–92.

Ruutiainen, J., Salonen, R., Halonen, P., Panelius, M., Eskola, J. & Salmi, A. (1991). Treatment of acute exacerbations in early multiple sclerosis: cyclosporin A or prednisolone? *Acta Neurol. scand.*, **83**, 52–54.

Ryffel, B. (1993). Cyclosporin binding proteins: identification, distribution, function and relation to FK binding proteins. *Biochem. Pharmac.*, **46**, 1–12.

The Multiple Sclerosis Study Group (1990). Efficacy and toxicity of cyclosporine in chronic progressive multiple sclerosis: a randomised, double-blinded, placebo-controlled clinical trial. *Ann. Neurol.*, **27**, 591–605.

Traugott, U. (1989). Detailed analysis of early immunopathologic events during lesion formation in acute experimental autoimmune encephalomyelitis. *Cell. Immun.*, **119**, 114–129.

Troiano, R., Cook, S. D. & Dowling, P. C. (1987). Steroid therapy in multiple sclerosis. *Archs Neurol.*, **44**, 803–807.

Trout, J. J., Koenig, H., Goldstone, A. D. & Lu, C. Y. (1986). Blood–brain barrier breakdown by cold injury: polyamine signals mediate acute stimulation of endocytosis, vesicular transport, and microvillus formation in rat cerebral capillaries. *Lab. Invest.*, **55**, 622–631.

Waage, A. & Bakke, O. (1988). Glucocorticoids suppress the production of tumour necrosis factor by lipopolysaccharide-stimulated human monocytes. *Immunology*, **63**, 299–302.

Pharmacological manipulation of blood-brain barrier (BBB) breakdown during recovery from experimental allergic encephalomyelitis (EAE) in the Lewis rat.

Paul, C. & Bolton, C. (1994) *J. Physiol.* **480P**, 11-12.

Table 1. % change in K_{aa} values for [^{14}C]-aminoiso-compared to control values

		Attack phase	Attack phase + cimetidine
CR-EAE	Brain	+ 95·38 %	+ 23·54 % †
	Spinal cord	+ 150·00 %	+ 42·00 % *
SFV	Brain	+ 74·20 %	+ 33·50 %
	Spinal cord	+ 40·84 %	+ 1·35 % ‡

$n = 3$–8 animals per group. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ compared to attack phase animals, unpaired t test.

There was a significant increase in BBB permeability in both CR-EAE and SFV compared to controls ($P < 0.001$). Table 1 shows that this increase in BBB permeability was significantly decreased by treatment with cimetidine. These results are consistent with a role for histamine in BBB opening in animal models of MS and that these effects are mediated via H_2 receptors. It is possible that preventing BBB permeability changes may be a useful therapeutic tool in MS.

REFERENCES

Butt, A.M. & Jones, H.C. (1992). *Brain Res.* 569, 100–105.
Egleton, R.D. & Segal, M.B. (1994). *J. Physiol.* 475.P, 71*P*.
Egleton, R.D., Butt, A.M., Amor, S. & Segal, M.B. (1994). *J. Physiol.* 479.P, 101*P*.

Pharmacological manipulation of blood–brain barrier (BBB) breakdown during recovery from experimental allergic encephalomyelitis (EAE) in the Lewis rat

C. Paul and C. Bolton

Pharmacology Group, School of Pharmacy & Pharmacology, University of Bath, Bath

The BBB is the highly selective vascular cell lining which maintains the homeostatic balance of the central nervous system (CNS), and loss of neurovascular function is a pivotal step in the pathogenesis of multiple sclerosis and the animal model EAE. The influx of unregulated plasma constituents and inflammatory cells into the CNS leads to characteristic oedema formation and myelin degradation. Previous studies have demonstrated enhanced permeability of the BBB during the acute phase of EAE in the Lewis rat and we have investigated the therapeutic effects of drugs to correct neurovascular disruption (Juhler *et al.* 1984; Paul & Bolton, unpublished work). Using a modification of the Leibowitz and Kennedy double radioisotope technique employing ^{125}I-rat serum albumin as the permeability marker and ^{111}In-red blood cells as the blood volume marker, we have extended our studies to examine neurovascular events during recovery following the loss of clinical signs and the resumption of body weight increases (Leibowitz & Kennedy, 1972). Neurovascular disruption, measured in the cerebellum, medulla-pons and cervical spinal tissues, continued in recovery. Interestingly, in comparison to extravascular blood equivalent (EVBE) values recorded in

acute EAE, permeability in the cerebellum was exacerbated during recovery (7·59 ± 2·1 in recovery, 3·02 ± 0·82 acute EAE, means ± S.E.M., $n > 5$) while spinal cord permeability was reduced (3·6 ± 0·63 in recovery, 9·58 ± 1·47 acute EAE, means ± S.E.M., $n > 5$). The ability of drugs to decrease BBB permeability was found to be augmented during the convalescent period. For example therapeutic administration of cyclosporin A (CsA) at 50 mg (kg body weight)$^{-1}$ (BW) partially corrected cerebrovascular damage (Paul & Bolton, unpublished work), but when administered in recovery returned BBB function to normal in all tissues ($P \leq 0.01$, Mann–Whitney U test (MWU)). Similarly, low dose CsA (25 mg (kg BW)$^{-1}$) and dexamethasone (0·1 mg (kg BW)$^{-1}$), given during acute EAE, reduced permeability in selected CNS tissues but demonstrated significant barrier improvements in all areas when given during recovery ($P \leq 0.05$ MWU). In summary, the BBB remains dysfunctional following complete recovery from EAE. Furthermore, pharmacological control of barrier function is improved compared with therapeutic drug efficacies. The findings suggest that mechanistic differences in barrier abnormalities exist between the acute and the recovery phases of EAE which may be important in the long-term treatment of neurological conditions.

REFERENCES

Juhler, M., Barry, D.I., Offner, H., Konat, G., Klinken, L. & Paulson, O.B. (1984). *Brain Res.* 302, 347–355.
Leibowitz, S. & Kennedy, L. (1972). *Immunology* 22, 859–869.

Role of neurochemicals in the blood–brain barrier permeability, cerebral blood flow, vasogenic oedema and cell changes in heat stress. Experimental observations in the rat

H.S. Sharma*, J. Westman†, F. Nyberg‡, J. Cervós-Navarro* and P.K. Dey§

**Institute of Neuropathology, Free University Berlin, Germany, Departments of †Human Anatomy and ‡Pharmaceutical Biosciences, Biomedical Centre, Uppsala University, Uppsala, Sweden and §Department of Physiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, India*

The possibility that serotonin, prostaglandins and neuropeptides are involved in the secondary mechanisms of cell damage in heat stress (HS) was examined in a rat model using pharmacological approach. Animals (80–90 g, 8–9 weeks old) were exposed to heat in a B.O.D. (biological oxygen demand) incubator for 4 h at 38 °C (relative humidity 45–50 %, wind velocity 20–25 cm s^{-1}) (Sharma *et al.* 1991) and hyperthermia, behavioural symptoms, blood–brain barrier (BBB) permeability (Sharma *et al.* 1992*a*), cerebral blood flow (CBF), brain oedema, and cell changes (Sharma *et al.* 1992*b*) were examined. In separate groups of animals ketanserin (a serotonin$_2$ receptor

Aspects of the biochemical pharmacology of neurovascular disruption in experimental allergic encephalomyelitis (EAE).

Bolton, C., Lees, P., Paul, C., Scott, G.S., Williams, K.I. & Woodyer, P. (1994) *J. Neuroimmunol.* 52(2), 113.

demonstrated that a murine IgG1 anti-class II MHC antibody (OX6) is able to both prevent and treat EAE in the Biozzi AB/H mouse. We have confirmed that this antibody is poorly cytotoxic, allowing mechanisms of action other than depletion of antigen presenting cells to be assessed. Despite its effect on disease, OX6 administration does not prevent priming of T cells in the draining lymph node, implying an alternative mechanism for regulation of the autoimmune disease process.

Modulatory action of essential fatty acids on experimental allergic encephalomyelitis (EAE)

L.S. Harbige, M.M. Morris, S. Amor
Department of Immunology, Rayne Institute, UMDS, St Thomas' Hospital, London, UK

Oral administration of linoleic, γ-linolenic, α-linolenic, or eicosapentaenoic acid from safflower, fungal, linseed and fish oil sources 7 days after neuroantigen immunisation had differential effects on the clinical signs of EAE in Lewis rats. γ-Linolenic acid-rich fungal oil at 500 mg/kg of body weight was fully protective, although at doses of 200 and 1000 mg/kg only delayed the onset of disease. Linoleic acid-rich safflower oil at 500, 750 and 1000 mg/kg decreased the severity of clinical EAE in a dose-dependent manner. α-Linolenic and eicosapentaenoic acid at 500 mg/kg of body weight prolonged the course of clinical EAE. The effects of these oils was also examined ex vivo in normal animals. Adhesion of lymphocytes from fungal oil treated rats to homologous brain endothelium was unaffected, although lymphocytes from safflower oil treated normal animals had 2–3-fold increase in adherence compared to lymphocytes from untreated animals. Both oil treatments enhanced the T-cell proliferative response to concanavalin A. Analysis by flow cytometry of splenic lymphocytes from oil treated rats indicated that $CD8^+$ but not $CD4^+$ cell surface determinants were affected. In addition, the $CD45^+$ determinant was affected by fungal but not safflower oil. This study suggests that oral administration of lipids have potent effects on EAE and may be useful in multiple sclerosis.

Aspects of the biochemical pharmacology of neurovascular disruption in experimental allergic encephalomyelitis (EAE)

C. Bolton, P. Lees, C. Paul, G.S. Scott, K.I. Williams, P. Woodyer
Pharmacology Group, School of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY, UK

Neuroimmune-associated cerebrovascular disruption is a characteristic feature of the experimental disease allergic encephalomyelitis (EAE). The precise mechanisms initiating blood–brain barrier breakdown are unknown but may require the production of permeability-inducing factors to trigger vascular injury. Candidate mediators of neuroendothelial damage include the potent vasodilator nitric oxide (NO) and polyamines (PA) which cause gross perturbation of endothelial cell membranes [1,2]. Preliminary investigations by us have shown significantly elevated levels of NO, measured as nitrate product, in CNS blood vessel isolates from Lewis rats with EAE. Our studies have also revealed an increase in the PA content of neurovascular preparations. Interestingly, short-term therapeutic steroid administration during early EAE inhibits NO and PA formation and restores the normal permeability to the CNS vasculature [3]. Therefore, down-regulation of neuroendothelial-derived permeability factor may account, in part, for the suppressive effects of steroid treatment on the course of EAE.

Koenig, H. et al. (1989) J. Neurochem. 52, 101–109.
Moncada, S. et al. (1991) Pharamacol. Rev. 43, 109–142.
Paul, C. and Bolton C. (submitted for publication) Int. J. Immunopharmacol.

Adhesion molecules and the binding of lymphocytes to cerebral blood vessel walls

A.J. Vora [a], D.C. Dumonde [a], M.E. Perry [b], K.A. Brown [a]
[a] *Immunology,* [b] *Anatomy Group, UMDS, St Thomas' Hospital, London SE1 7EH, UK*

Antibody blocking studies and immunocytochemical techniques were used to identify the adhesion molecules that promote the attachment of blood lymphocytes to vessel walls in sections of human brain. Lymphocyte binding was impaired ($P < 0.02$) by anti-LFA-1 (46% inhibition) and anti-VLA-4 (mean 26% inhibition) antibodies. Counter-receptors for LFA-1 and VLA-4 are ICAM-1 and VCAM-1 and they were present on 48% and 27% of exposed blood vessels, respectively. $CD4^+$ T cells were the most adherent of the lymphocyte subsets and lymphocyte binding was augmented following activation with IL-2 (mean 130% increase; $P < 0.002$). These results should benefit the development of agents to impede lymphocyte entry across the blood–brain barrier.

This work was supported by The Henry Smith's Charity.

Restoration of blood-brain barrier integrity by dexamethasone and cyclosporin A combined dose therapy during experimental allergic encephalomyelitis.

Paul, C. & Bolton, C. (1994) *J. Neuroimmunol.* **54**(1-2), 188.

W17.05

RESTORATION OF BLOOD-BRAIN BARRIER INTEGRITY BY DEXAMETHASONE AND CYCLOSPORIN A COMBINED DOSE THERAPY DURING EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

C. Paul & C. Bolton
Pharmacology Group, School of Pharmacy & Pharmacology, University of Bath, Bath BA2 7AY, U.K.

Introduction: A focal point in pathogenesis of multiple sclerosis and the animal model experimental allergic encephalomyelitis (EAE) is the breakdown of blood-brain barrier (BBB) integrity. In EAE the abnormal BBB permeability can be reduced dose-dependently by therapeutic administration of either dexamethasone (Dex) or cyclosporin A (CsA). We now present evidence of enhanced drug action at the BBB when Dex and CsA are used in combination.
Materials and Methods: Drugs were administered for 2 days following symptom onset; Dex was injected sub-cutaneously twice daily in PBS; CsA in olive oil was given orally once daily. Albumin extravasation in the cerebellum, medulla-pons and cervical spinal cord was assessed by a modified double radioisotope technique.
Results: CsA in combination with 0.1mg/Kg Dex, a dose of minimal therapeutic benefit, demonstrated enhanced restoration of BBB function at 50, 35 and 25mg/Kg. Improved correction of barrier permeability was also seen when 50mg/Kg CsA was used in conjunction with 0.01mg/Kg Dex.
Conclusion: Low dose Dex enhanced the restorative action of CsA at the BBB. Therapeutically effective low dose combinations of Dex and CsA may result in fewer side effects and could therefore have important implications in the treatment of multiple sclerosis.

Immunomodulation of nitric oxide (NO) and its contribution to neurovascular permeability during experimental allergic encephalomyelitis (EAE).

Scott, G.S., Paul, C., Whitehouse, J., Williams, K.I. & Bolton, C. (1995) *Inflammation Res.* **44**(Suppl.3), S233.

W03/07

Immunomodulation Of Nitric Oxide (NO) And Its Contribution To Neurovascular Permeability During Experimental Allergic Encephalomyelitis (EAE)

GS. Scott, C. Paul, J. Whitehouse, KI. Williams & C. Bolton
Pharmacology Group, University Of Bath, Bath BA2 7AY

Our initial studies demonstrated that the loss of cerebrovascular integrity in the animal model of multiple sclerosis, EAE can be suppressed by dexamethasone (Dex) and cyclosporin A (CsA). The mechanisms through which the drugs inhibit neurovascular breakdown are unclear but may involve a dual action on circulating inflammatory cells and the cerebroendothelium. The current study has shown that Dex but not CsA alters the circulating inflammatory cell composition in Lewis rat EAE. However, both agents reduced the levels of NO within central nervous system components. NO acts as a potent vasodilator and cytotoxic molecule, which is produced by inflammatory cells, and may contribute to neurovascular disruption during EAE. The corrective effects of Dex on the cerebrovasculature may therefore be mediated through suppression of NO or redistribution of inflammatory cells, whereas the effects of CsA may be elicited through NO inhibition..